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The Effect of Nutritional Stress on the Reproductive Behaviors of Females and their Offspring in Meadow Voles, Microtus pennsylvanicus

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THE EFFECT OF NUTRITIONAL STRESS ON THE REPRODUCTIVE BEHAVIORS OF FEMALES AND THEIR OFFSPRING IN MEADOW VOLES, *MICROTUS PENNSYLVANICUS*

by

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ABSTRACT

Sabau, Ramona Magdalena. Ph.D. The University of Memphis. May 2014. The effect of nutritional stress on reproductive behavior of females and their offspring in meadow voles, (*Microtus pennsylvanicus*). Major Professor: Michael H. Ferkin, PhD.

Many female small mammals face limited food availability during pregnancy, postpartum estrus and/or lactation. The amount of food that is available to these females may influence their behavior and reproductive success. My research examined the response of female meadow voles in different reproductive states when faced with nutritional stress in terms of reproductive behaviors (sexual and maternal behavior) and how maternal nutrition and/or maternal care can affect individual variation in some aspects of offspring phenotype. I found that during PPE, females that were FD or FR during late pregnancy were sexually less receptive and produced scent marks that were no longer as attractive as those produced by control PPE females. FD but not FR caused females to no longer display preferences for the scent marks of males (proceptivity). I also discovered that dams that were not FR spent more time engaged in maternal behavior during lactation than compared to dams that were food restricted at this time. Dams that were FR during days 8-14 of lactation displayed the most pronounced decline in maternal behavior relative to dams that were restricted during days 1-7 or days 15-21 of lactation. Lastly, I found that FR during days 8-14 of lactation induced deficits in sexual behavior and body mass of male and female offspring at puberty and later as adults. A reduction in maternal care or the food restriction experienced by the pups singly or together may be sufficient to trigger persistent, sex-specific effects on the body mass and sexual behavior of male and female meadow voles similar to those observed in other rodents.

PREFACE

The papers included in this dissertation have been written in the format of the professional journal in which they have been either submitted or published. Each chapter has been written in a way that allows for interpretation independent of the other components of this dissertation.

Chapter 1: table of contents and list of figures: formatted according to the style of *Ethology*.

Chapter 2: formatted according to the style of Ethology. Publication appeared as: Sabau & Ferkin 2013. Food deprivation and restriction during late lactation affects the sexual behavior of postpartum estrus female meadow voles, *Microtus pennsylvanicus*. *Ethology* 2012; 119(1):10.1111/eth.12033.

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Chapter 4: Submitted with formatting according to the style of *Ethology*.

Chapter 5: Submitted with formatting according to the style of *Behavior*.

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CHAPTER 1

INTRODUCTION

Many female small mammals may be limited to the amount of food in their territory (Batzli 1985). Nutritional stress may be an environmental challenge faced by female meadow voles, when they are in different reproductive states: non-pregnant, pregnant, postpartum estrus (PPE) or lactating. Thus, food availability may affect a female vole's sexual behavior. Sexual behavior in female mammals can be separated and characterized into three observable components: attractivity, proceptivity, and receptivity (Beach 1976). Attractivity refers to the relative stimulus values of cues and signals of different senders of the same sex, when assessed by receivers of the opposite sex (Beach 1976). Proceptivity refers to the sexually-appetitive responses of receivers to cues and signals of opposite-sex conspecifics, senders (Beach 1976). Attractivity and proceptivity establish communication between potential mates, and allow them to coordinate behaviors that facilitate or inhibit direct interactions (Beach 1976; Johnston 1979; Stopka & Macdonald 1998). Receptivity refers to a female's willingness to mate (Beach 1976) and includes mounts, intromissions, thrusts, and ejaculations (Gray & Dewsbury 1975; delBarco-Trillo & Ferkin 2004).

During lactation, female mammals are faced with higher energy demands associated with rearing their litter (Gittleman & Thompson 1988; Mattingly & McClure 1982; Migula 1969) and sometimes are facing with food shortage in their territory (Batzli 1985) before and/or after giving birth. This may cause females that are lactating to adopt 1 of 3 possible strategies to deal with a food shortage (Konig 1989; Perrigo 1987, 1990;

Rauw et al. 2003; Therrien et al. 2007). First, dams could reduce the size of their current litter but maintain high levels of maternal care. By doing so, dams would not be sacrificing their future parental investment (Trivers 1972). Second, dams may maintain the size of their current litter but show a reduction in the maternal care they provide. Third, dams may maintain litter size and maintain high levels of maternal care. In this way, dams would be sacrificing their body condition and future parental investment (Trivers 1972). An animal's nutritional state my play a critical role in expression of sexual and maternal behavior, the most important behaviors that influence reproductive success of individuals. Deficits and decreases in these behaviors may reduce the mating and reproductive success of male and female meadow voles (Boonstra et al. 1993; Berteaux et al. 1999), which may impact population demography (Tamarin et al. 1984; Desy & Batzli 1989).

In many species of small mammals, including meadow voles, *Microtus pennsylvanicus*, females come into postpartum estrus (PPE) within 12-24 h of giving birth, allowing them to mate and become pregnant while raising the current litter. PPE females show increases in attractivity, proceptivity, and receptivity, the three components of sexual behavior, relative to females not in PPE (Dewsbury et al. 1979; Gilbert 1984; Witt et al. 1990). Previous work has shown that in non-pregnant, non-lactating females, the three components of sexual behavior can be affected by alterations in the amount of food available to a female (Bronson 1989; Wade et al. 1996; Pierce et al. 2005). We tested the hypothesis that food deprivation (FD) and food restriction (FR) during late gestation causes deficits and decreases the attractivity, proceptivity, and receptivity of females when they enter PPE. Specifically, we predicted that such female voles will 1)

no longer produce scent marks that are attractive to male conspecifics (attractivity), 2) no longer show a preference for the scent marks of male conspecifics to those of female conspecifics (proceptivity), and 3) no longer mate (increased receptivity) immediately after giving birth, when they enter postpartum estrus.

Many female small mammals face limited food availability during lactation. These dams may have to choose between altering the amount of maternal behavior they provide to their young, reducing the size of their litter, or not adjusting their behavior or litter size. How females allocate energy to maternal investment may depend on the energy costs of different lactation stages. Thus, females kept on a restricted diet during early lactation may not display the same changes in maternal behavior as females restricted during middle or late lactation. We hypothesized that the amount of time female voles provided maternal behavior would differ if they were food restricted during early, middle, or late in lactation. We tested this hypothesis by placing lactating female meadow voles, *Microtus pennsylvanicus,* into 1 of 4 groups: dams that underwent a 30% caloric restriction during days 1-7 of lactation, those that were food restricted on days 8-14, those that were food restricted on days 15-21, and dams that did not undergo food restriction during lactation.

Individual differences in phenotype have received much attention in the literature, however, little is known about the proximate causes of this variation (Forstmeier et al. 2004). Recently, the adaptive significance of maternal effects has been widely recognized as a mechanism for adaptive phenotypic response to environmental heterogeneity (Bernardo 1996; Mousseau & Fox 1998). The environment experienced by pups during lactation (nutrition and maternal behavior) can contribute not only to sexual

development, but also to individual differences in offspring sexual behavior. Our goal in chapters 4 and 5 was to determine whether maternal food restriction can have persistent effects on body mass, food intake and sexual behavior of offspring when they reach adulthood. We tested the hypothesis that the offspring of meadow vole dams, *Microtus pennsylvanicus*, that were 30% food restricted (FR) during days 1-7 of lactation (FR 1-7), days 8-14 of lactation (FR 8-14), or days 15-21 of lactation (FR 15-21) show negative effects on their food intake, growth, and the three components of sexual behavior (attractivity, proceptivity, and receptivity) compared to those offspring of control dams. We predicted that these effects would be more pronounced in the FR 1-7 and FR 8-14 offspring than in the FR 15-21 or control offspring. We tested this hypothesis and prediction by measuring the three components of sexual behavior: attractivity, proceptivity/interest in the opposite sex, and receptivity (Beach 1976).

Overall, my research focused on testing hypotheses that examined the effects of food restriction or deprivation before and during the different stages of lactation. My efforts will increase our understanding of these effects.

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CHAPTER 2

Food deprivation and restriction during late gestation affects the sexual behavior of postpartum female meadow voles, *Microtus pennsylvanicus*

INTRODUCTION

Sexual behavior in female mammals can be separated and characterized into three observable components: attractivity, proceptivity, and receptivity (Beach 1976). Attractivity refers to the relative stimulus values of cues and signals of different senders of the same sex, when assessed by receivers of the opposite sex (Beach 1976). Proceptivity refers to the sexually-appetitive responses of receivers to cues and signals of opposite-sex conspecifics, senders (Beach 1976). Attractivity and proceptivity establish communication between potential mates, and allow them to coordinate behaviors that facilitate or inhibit direct interactions (Beach 1976; Johnston 1979; Stopka & Macdonald 1998). For most terrestrial mammals, including rodents, these interactions depend on olfactory signals, such as scent marks, from the sender and the responses of receivers to these scent marks (Brown 1985; Johnston 2003; Ferkin et al. 2004). Receptivity refers to a female's willingness to mate (Beach 1976). Mating includes mounts, intromissions, thrusts, and ejaculations (Gray & Dewsbury 1975; delBarco-Trillo & Ferkin 2004).

The three components of sexual behavior can be affected by altering the amount of food available to a female (Bronson 1989; Wade et al. 1996; Pierce et al. 2005). For example, food-restricted or food-deprived female rats (*Rattus norvegicus*), musk shrews (*Suncus murinus*), house mice (*Mus musculus*), Syrian hamsters (*Mesocricetus auratus*), and voles (*Microtus* spp.) were less attractive to male conspecifics and often failed to show interest in responding to potential mates, and, if given an opportunity to mate, they

usually did not mate (Bronson & Marstellar 1985; Wade & Schneider1992; Gill & Rissman 1997; Jones & Wade 2002; Temple et al. 2000; Pierce et al. 2005). Typically, 48-72 hours of food restriction and 24-48 hours of food deprivation were sufficient to induce deficits in the sexual behavior of these female small mammals (Gill & Rissman 1997; Jones & Wade 2002; Temple et al. 2002; Pierce et al. 2005). However, it should be noted that these studies were carried out on female subjects that were not pregnant or lactating. We do not know if females that undergo food restriction or food deprivation during late gestation display similar deficits and decreases in their sexual behaviors when they later enter postpartum estrus.

For numerous species of terrestrial mammals, postpartum estrus, PPE, is a period of heightened attractivity, proceptivity, and receptivity that occurs shortly after the dam delivers her litter (Dewsbury et al. 1979; Gilbert 1984; Witt et al. 1990). Previous studies have shown that PPE females, which were not food restricted or food deprived during gestation, produced odors and scent marks that were more attractive to males than were those produced by females not in PPE (Ferkin & Johnston 1995; Zeigler et al. 1993; Lai et al. 1996; Lazaro-Perea et al. 1999; Vaughn et al. 2011). PPE females also spent more time and show increases relative to females not in PPE in scent marking, over-marking, and self-grooming when they encounter the scent marks of males (Ferkin et al. 2004; Ferkin 2006). PPE females were more likely than females not in PPE to attract mates and to signal their interest in them (Witt et al. 1990; Rudd 1994; Ferkin 2006). PPE females also mated more readily and get pregnant more often than did females not in PPE (Gilbert 1984; Keller 1985; delBarco-Trillo & Ferkin 2007). Thus, PPE females preferred as

mates by male conspecifics (Ferkin et al. 2008). However, these females may no longer be preferred mates if they had undergone nutritional stress during gestation.

Nutritional stresses such as food deprivation and food restriction may be an ecological challenge faced by small herbivores, such as meadow voles, *Microtus pennsylvanicus*. Meadow voles live in ephemeral and transitional grasslands, where food sources are patchy and vary in quality across the territories of female conspecifics (Getz 1985; Bergeron & Jodoin 1987; Bergeron et al. 1990). As females enter late gestation they become relatively sedentary (Madison 1980a, b, 1985) and may be limited to the forage that is available in their territory (Batzli 1985). Consequently, females during late gestation may become food restricted or food deprived.

In this study, we test this hypothesis that food restriction or food restriction during late gestation is sufficient to induce postpartum estrus female meadow voles to manifest deficits and decreases in the three components of sexual behavior. Specifically, we predicted that such female voles will 1) no longer produce scent marks that are attractive to male conspecifics (attractivity), 2) no longer show a preference for the scent marks of male conspecifics to those of female conspecifics (proceptivity), and 3) no longer mate (receptivity) immediately after parturition, when they enter postpartum estrus. Deficits and decreases in sexual behavior may reduce the mating and reproductive success of male and female meadow voles (Boonstra et al. 1993; Berteaux et al. 1999), which may impact population demography (Tamarin 1977; Tamarin et al. 1984; Desy & Batzli 1989).

METHODS

Animals

The meadow voles used in the study were 4^{th}-6^{th} generation captive animals born and raised in a room that was maintained between 23-25 degrees C and under long photoperiod (14:10 h light: dark, lights on at 0700 hours, C ST). This photoperiod simulates the day length during the breeding season in free-living meadow voles (Zucker et al. 1980). All the female meadow voles used in the study were between 120-150 days of age, similar in body weight to one another (within 3-5 grams) , and sexually experienced, having previously delivered a litter, when they were 70-80 days of age, Female meadow voles are induced ovulators and do not undergo estrous cycles (Keller 1985). Male meadow voles were between 120-150 days of age, similar in body weight to one another (within 3-5 grams), and sexually experienced, having previously sired a litter when they were 70-80 days of age. Males and females had continuous access to food prior to their inclusion in the study. We paired 40 males with 40 females for three days in breeding cages (37×21×15 cm; l, w, h). After 24 hours, the males were removed from the breeding cages and returned singly to their home cages in the main colony. We adhere to the 'Guidelines for the use of animals in research' as published in Animal Behaviour (1991, 41, 183-186) and the laws of the country where the research was conducted.

Treatment Groups

We were able to determine by tactile palpation which females were pregnant 10-14 days after pairing. Of the 40 pairs of voles that were allowed to mate, 36 pairs mated and later produced litters. Thus, we were able to assign pregnant females to three equal-

sized groups. Females in group 1 (control) had continuous access to food during gestation. Females in group 2 had continuous access to food before day 18 of gestation, but were provided with 70% of the daily intake of control animals between day 18 and 20 of gestation, the last three days of gestation (FR). Females in group 3 had continuous access to food until day 20 of pregnancy, when they were food deprived for 24 h before expected parturition (FD). Previous studies have showed that 48-72 hours of food restriction and 24-48 hours of food deprivation were sufficient to induce deficits in sexual behavior of female small mammals that were not pregnant or lactating (Gill & Rissman 1997; Jones & Wade, 2002; Temple et al. 2002; Pierce et al. 2005). We chose to use similar durations of food restriction and deprivation during gestation to determine whether female voles in poor nutritional condition display the heightened sexual behaviors characteristic of postpartum estrus. We selected a 30% food restriction of what control PPE females consumed. We did so, because this may be typical of what a female vole may encounter in the field (Batzli 1985), and because our results from a pilot study showed that all dams survived and none cannibalized young when they underwent such a food restriction during the last 3 days of gestation.

In addition, we used 40 females from our colony that had continuous access to food, and were not currently pregnant or lactating; these females were in behavioral estrus and designated as reference (REF) females (Ferkin & Johnston 1995; Vaughn et al. 2011). We compared the sexual behavior of REF females to that of females in groups 1-3.

Attractivity

Scent Donors and Subjects

Forty-eight females were selected to be scent donors. Donors were placed on the dietary regimens detailed above so that there were 12 females in each group: 12 REF females, 12 control PPE females, 12 FR females, and 12 FD females. Subjects were 48 male voles that had continuous access to food. Males were randomly chosen from a pool of sexually experienced male voles that were unrelated to and unfamiliar with the female scent donors used in the attractivity tests. We did not use more than two individuals from the same litter in any experimental group to eliminate the potential for litter effect. We used a Latin Squares design to allow PPE females to serve as scent donors in the attractivity tests and then as subjects in the proceptivity tests and receptivity tests (Pierce et al. 2005). That is, some PPE females were subjects in the proceptivity first, some were first subjects in receptivity tests, and others were first used as donors in attractivity tests. We used female subjects, male studs, and male and female scent donors were unfamiliar and unrelated to the voles with which they were tested.

Testing procedure

Each male underwent a single 5-minute attractivity test that followed the procedures detailed by Pierce and colleagues (Pierce & Ferkin 2005; Pierce et al. 2005, 2007). Briefly, we recorded the amount of time in seconds that males spent investigating the anogenital area scent marks of the following pairs of female scent donors: 1) a FR PPE female versus a PPE female that was not food restricted (PPE); 2) a FD PPE female versus a PPE female that was not food deprived; 3) a FR PPE female versus a REF

female; 4) a FD PPE female versus a REF female; 5) a FR PPE female versus a FD PPE female. We used matched-paired t-tests (two tailed) to determined whether significant differences existed in the amount of time each male subject spent investigating the scent marks of the females in each paired comparison. All significant differences were accepted at $\alpha = 0.05$. Each male subject was tested once and with a unique pair of female scent donors.

The test involved male subjects being presented with a clean microscope slide (2.5×7.6 cm) that contained an anogenital area scent mark from two different female scent donors (Pierce et al. 2005). Each slide was divided in three equal sections; each section was 2.5 cm long. One end section contained a scent mark from one female donor, while the other end section contained a scent mark from the other female donor. The middle section contained no stimulus odor. The glass slide was suspended by a wire hook and a clasp 1 cm above the substrate in the subject's home cage, against the wall opposite the animal's nest. During each 5-minute trial, we recorded continuously the time each subject investigated each section of the slide. Criteria for investigation of a mark were that the male subject 1) licked or sniffed a stimulus odor or its nose was within 1 cm of one end of the slide, 2) investigated both of the two scented areas on the slide, and 3) spent more time investigating the two scented areas of the slide than the clean middle section (Pierce et al. 2005). The test began when the slide was placed into the cage of subject. Each slide was used in only one trial and discarded. We randomly placed the stimulus odors on the left or the right side of the slide.

Fresh scent marks were obtained for each trial from each scent donor. We collected scent marks from the anogenital area by rubbing a clean microscope slide

against it for 5-10 seconds. The anogenital area may contain components of scent from multiple sources such as urine, feces, sex organs, and sebaceous glands but are responded to similarly by conspecifics (Ferkin & Johnston 1995); the anogenital area scent marks are deposited by voles in their runways (Ferkin et al. 2004). The experimenter wore disposable latex gloves to minimize human scent transfer while handling all slides. All testing occurred within 12 hours of the pregnant females delivering their litters.

Proceptivity

We used a scent preference test to determine whether nutritional stress induced before parturition induced differences in proceptive behavior of PPE females. Scent donors were 36 REF females and 36 sexually experienced males that had not been paired with any of the subject females and had not been used as subjects for the attractivity tests. The subjects were the 36 females from diet-treatment groups 1-3. Each subject was tested once with a unique pair of male and female scent donors.

We followed the methods for proceptivity testing developed by Pierce and collaborators (Pierce et al. 2005) and are similar to the attractivity test we described above, with one notable exception. In the proceptivity test, females are exposed to a glass slide that contains the scent mark of a REF female and the scent mark of an unfamiliar male conspecific. During the 5-minute long proceptivity tests, we recorded the amount of time subject females in each group investigated the end of the slide containing the scent mark of the male scent donor and the end containing the scent mark of the female scent donor. Female voles were considered to display proceptive behavior if they spent

significantly more time investigating the odors of a male conspecific than those of a female conspecific (Pierce & Ferkin 2005; Pierce et al. 2005, 2007). Statistical analyses followed those used for the attractivity test.

Receptivity

Subjects were 36 female voles from diet-treatment groups 1-3 and 36 male voles; the male voles were not used in the attractivity and the proceptivity tests. We used the same methods in testing receptivity as described by Pierce et al. (2005). Briefly, each stud male was paired with a single female from one of the dietary groups in a clear, plastic cage (37×21×15 cm; l, w, h), containing hardwood shavings, bedding, nesting material and water. Pups were kept with the dam in the cage until weaning on day 18; no pups were attacked or injured.

Introduced pairs were allowed to interact for 4 hours; voles typically mate within this time 4-hour period (delBarco-Trillo & Ferkin 2007; Vaughn et al. 2008, 2011). We recorded each 4-hour paring (Sony Handycam DCR-SR68). During playback, we scored the number of ejaculations by each male and his latency to first ejaculation, which was the amount of time (seconds) that elapsed between the male's introduction into the female's cage and his first ejaculation. We used Chi-Square tests to compare the number of females that mated in the PPE control group with the numbers of females that mated in the food-restricted group and those that mated in the food-deprivation group. We used the number of females that mated in the control PPE group as the expected value for the Chi-Square tests. We used two separate one-way ANOVA's to determine if males paired with

control PPE females, food-restricted females, or food-deprived females differed in their number of ejaculations and latency to first ejaculation.

RESULTS

Effects of food restriction and food deprivation on attractivity

Food restriction and food deprivation during late gestation was sufficient to induce day 1 lactating (PPE) females to no longer produce scent marks that were as attractive as those of control PPE females to males. Male voles spent more time investigating the scent mark of control PPE females than that of FD females (t_{11} =12.01, $p < 0.001$; Fig. 1) as well as the scent marks of FR females (t_{11} = 3.76, p= 0.003; Fig. 1). Food restriction and food deprivation during late gestation was sufficient to induce PPE females to produce scent marks that were as attractive as those of REF females to males. Male voles spent similar amounts of time investigating the scent marks of REF females compared to the scent marks of FD females (t_{11} = 0.485, p = 0.63) and the scent marks of FR females (t_{11}= 1.799, p = 0.099; Fig. 1). Males also spent similar amounts of time investigating the scent mark of a FR female and that of a FD female (t_{11}= 0.892, p =0.39).

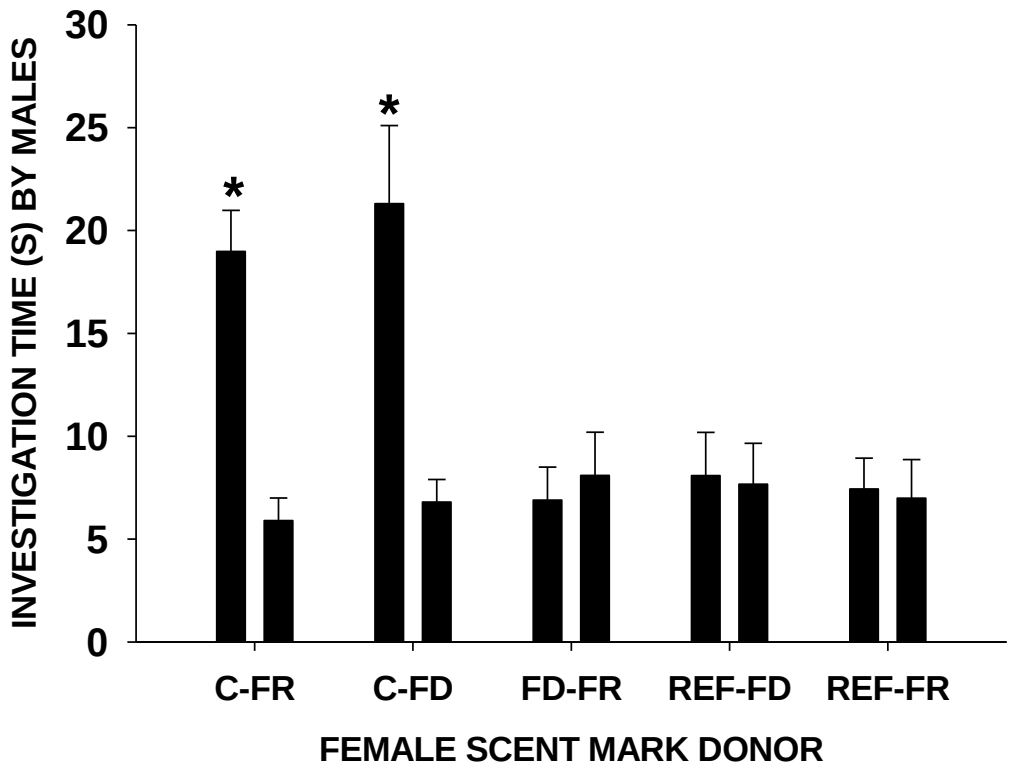

Figure 1. Mean ± SEM time (seconds) during the 5-minute long attractivity tests that male meadow voles spent investigating the anogenital scent marks of 1) control (C) females, PPE females that had continuous access to food and those that were food restricted for 3-4 days before giving birth (FR), 2) control PPE females and females that were food deprived for 24-48 hours before giving birth (FD), 3) FD females and FR females, 4) females that were not pregnant and lactating and that had continuous access to food (REF) and FD females, and 5) REF females and FR females. * Asterisk indicates significant differences between pairs at $p < 0.001$.

Effects of food restriction and food deprivation on proceptivity

We found that food restriction of 30% of the intake of control PPE females resulted in no effect on our measure of proceptivity. FR PPE females spent more time investigating scent of males than those of females ($t_{11} = 2.610$, $p = 0.02$; Fig. 2). FD PPE females, spent similar amounts of time investigating the scent of opposite-sex and same-sex conspecifics ($t_{11} = 0.138$, $p = 0.89$; Fig. 2), suggesting an inhibition of proceptive behavior. Control PPE females spent more time investigating the odors of opposite sex conspecifics ($t_{11} = 4.68$, $p < 0.001$; Fig. 2).

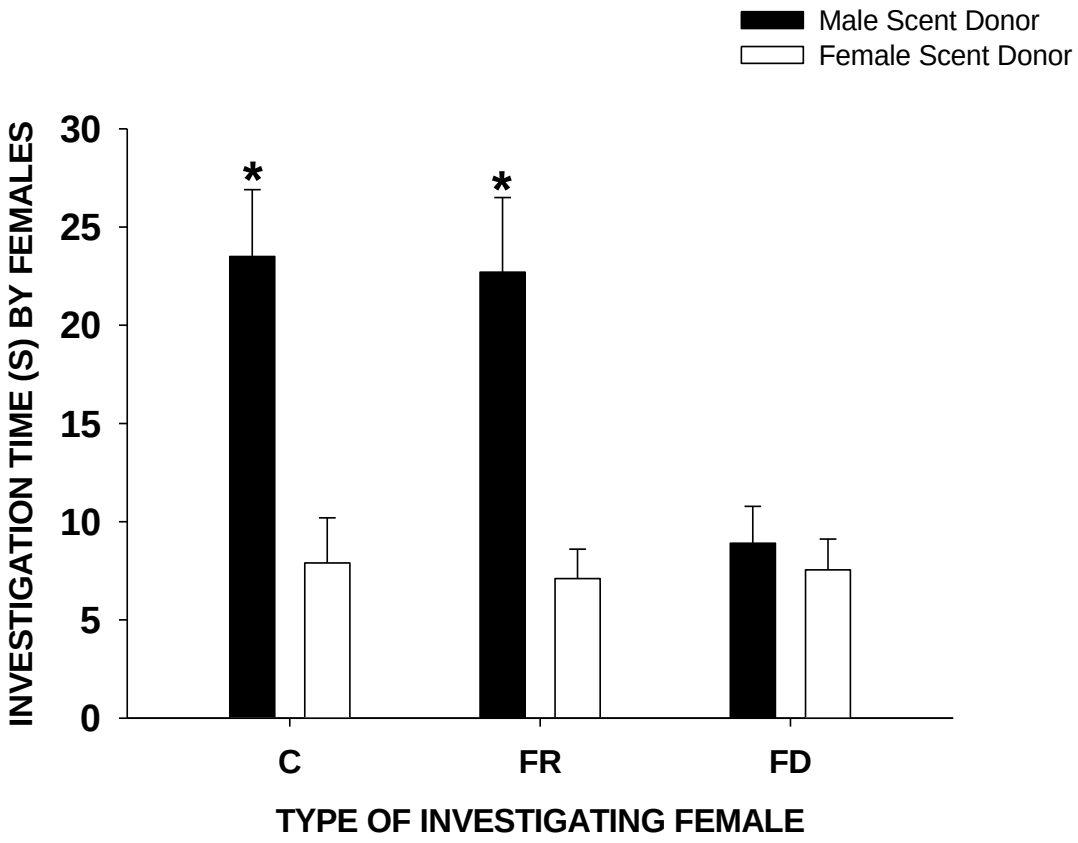

Figure 2 Mean ± SEM time (seconds) during the 5-minute long proceptivity tests that control PPE females (C), food-restricted females (FR), and food-deprived females (FD) spent investigating the anogenital scent mark of a male conspecific and that of a female conspecific. * Asterisk indicates significant differences between pairs at $p < 0.001$.

Effects of food restriction and food deprivation on receptivity

We found that 18 females, 11 of 12 females from the control PPE group, 4 of 12 females from the food-restricted group, and 3 of 12 females from the food-deprived group mated when paired with an unfamiliar, male conspecific. The number of food-deprived and the food-restricted females that mated were significantly different from that of the control PPE females that mated (X^2 =4.45, df = 1, p = 0.035 and X^2 = 5.81, df = 1, p = 0.016, respectively). Of the females that mated, those in the control PPE group, those

in the food-restricted group, and those in the food-deprived group did not differ in our measures of copulatory behavior. The number of ejaculations received by control females, food-deprived females, and food-restricted females were similar ($F_{2, 12} = 0.68$, $p= 0.52$; Fig. 1.3). There were also no significant differences in the latency to first ejaculation ($F_{2, 12} = 1.42$, $p= 0.27$) for males paired with control females (mean ± SEM, 2890.4 ± 235.1 seconds), food-deprived females (3050.3 ± 319.3 seconds), or food-restricted females; (3353.7 ± 372.6 seconds).

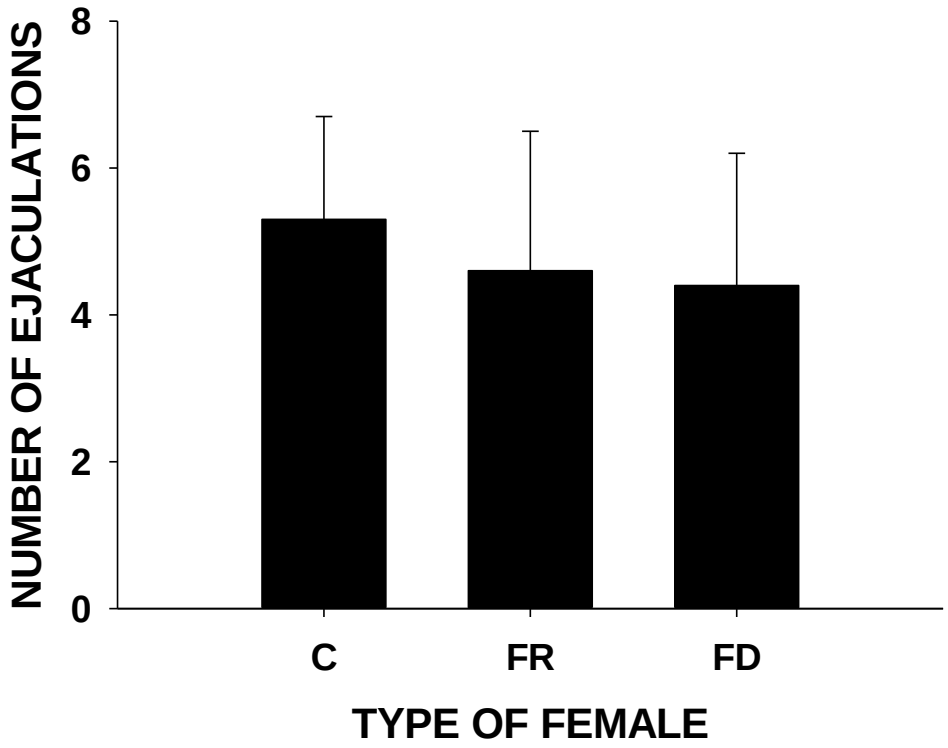

Figure 3 Mean ± SEM number of ejaculations by stud males during the 4-hour receptivity tests when paired with a control PPE female (C), a food-restricted PPE female (FR), or a food-deprived PPE female (FD). There were no significant differences between groups.

DISCUSSION

Female meadow voles that underwent either food restriction or food deprivation during late gestation showed deficits and reductions in their sexual behavior (attractivity, proceptivity, and receptivity during postpartum estrus. Food deprivation on day 20 of gestation, the day before parturition, and 30 % food restriction starting on day 18 of gestation was sufficient to induce female meadow voles on day 1 of lactation, when the females would enter PPE, to produce anogenital area scent marks that were was not as attractive as those produced by control PPE females. Food-deprived female voles also produced odors that were less attractive than those of control PPE females to males. We also found females that were food deprived or food-restricted produced scent marks that were as attractive as those produced by REF females; females that are not pregnant, lactating or in PPE. Previous work has shown that REF females produce odors that are less attractive than those produced by PPE females to males (Ferkin & Johnston 1995; Vaughn & Ferkin 2011). Our data suggest that PPE females in poorer nutritional states may not be attractive to potential mates. These results also suggest that male voles can distinguish between the scent marks of females in different reproductive and nutritional states, using both of these condition-dependent features to assess the scent marks of potential mates.

We found females that were food restricted during late gestation and control females entered postpartum estrus and maintained their preferences for the scent marks of male conspecifics over those of female conspecifics. In contrast, females that were food deprived during late gestation no longer expressed a preference for the scent marks of male conspecifics; food-deprived females spent similar amounts of time investigating the

scent of males or female voles. Similarly, Pierce et al. (2005) found REF female voles that were food deprived for six hours spent similar amounts of time investigating the scent marks of male and female conspecifics. Taken together, our findings suggest that food-restricted females may still be motivated or interested in locating potential mates, whereas food-deprived females may not be as motivated or interested in locating potential mates. The decline in proceptive behaviors among food-deprived females may reflect the fact these females have to rely entirely on limited energetic reserves for their maintenance and that of their pups; small mammals such as voles can survive only 2-3 days without food (Bronson 1989). Typically, voles eat small meals throughout the day (Batzli 1985). A 30% food restriction may not cause dams to rely entirely on stored energy to meet the high costs of entering postpartum estrus and lactation. Thus, 30% food-restricted females may be more inclined to seek out mates during postpartum estrus than would food-deprived females, who may have used their remaining energy stores to support their current litter.

Our results show food deprivation and food restriction during late gestation affected a female vole's sexual receptivity postpartum. Eleven of 12 control PPE females (92%) copulated. Several studies have shown that nearly all female voles that enter PPE mate (Keller 1985; Witt et al. 1990; delBarco-Trillo 2007). We discovered that only 3 of 12 food-deprived dams (25%) and 4 of 12 of the food-restricted dams (33%) copulated during the receptivity test. Interestingly, fewer food-deprived females mated than did food-restricted females, suggesting that food deprivation may have more potent effects than food restriction on inhibiting sexual receptivity; this is similar to the pattern of our results for our test of proceptivity. We know of no comparable studies that have

examined the effects of food deprivation on sexual behavior of postpartum estrus females. However, female mammals that were not lactating or in postpartum estrus also respond to food deprivation by reducing their copulatory behavior or willingness to mates (Wade et al. 1996; Gill & Rissman 1997). For example, Pierce et al. (2005) found that six hours and 24 hours of food deprivation were sufficient to inhibit sexual receptivity in 8 of 9 females and 10 of 10 females that were not pregnant or lactating (REF females in our study), respectively.

Interestingly, the nutritional state of the dams did not affect our measures of copulatory behavior. The latency to mate was similar for all females that mated in our study. In addition, dams received between 4-6 ejaculations from their paired males, independent of whether she was a control, food deprived, or food restricted during late gestation. The number of ejaculations per copulatory bout reported in this study also matched those reported in meadow voles that were exposed to different risks of sperm competition (Gray & Dewsbury 1975; delBarco-Trillo & Ferkin 2004; Vaughn et al. 2008, 2011). This suggests that the number of ejaculations by male meadow voles during a copulatory bout may be independent of the context.

Our findings suggest that most female meadow voles facing food restriction or food deprivation late in gestation do not enter into a heightened state of sexual behavior. In that more than 95% of free-living females voles mated during PPE (Tamarin 1977; Keller 1985; McShea & Madison 1989), and dams may be exposed to food shortages during gestation (Batzli 1985; Bergeron & Jodoin 1987; Bergeron et al. 1990), dams that are food restricted or food deprived late in gestation may not display the heightened sexual behaviors associated with postpartum estrus. In doing so, nutritionally-challenged

dams may forgo the benefits of mating during PPE to meet the increased energetic costs of her survival and that of her current litters (Trivers 1972). Such a response by food-deprived and food-restricted females may reduce the number of pups that they may deliver in their life span (Tamarin 1977), which could affect population demographics (Tamarin et al. 1984; Desy & Batzli 1989).

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CHAPTER 3

Food restriction affects the maternal behavior provided by female meadow voles, *Microtus pennsylvanicus*

INTRODUCTION

During lactation, female mammals are faced with high energy demands associated with rearing their litter (Gittleman and Thompson 1988; Mattingly and McClure 1982; Migula 1969). Many female small mammals may be limited to the amount of food in their territory (Batzli 1985). This may cause females that are lactating to adopt 1 of 3 possible strategies to deal with a food shortage (Konig 1989; Perrigo 1987, 1990; Rauw et al. 2003; Therrien et al. 2007). First, dams could reduce the size of their current litter but maintain high levels of maternal care. By doing so, dams would not be sacrificing their future parental investment (Trivers 1972). Second, dams may maintain the size of their current litter but show a reduction in the maternal care they provide. Third, dams may maintain litter size and maintain high levels of maternal care. In this way, dams would be sacrificing their body condition and future parental investment (Trivers 1972). A review of the literature suggests that no consensus exists on which strategy a lactating female rodent adopts when they face a food shortage. Some studies reported that female rats that are food-restricted , *Rattus norvegicus*, spent less time involved in maternal behavior (Smart and Preece 1973; Smart 1976), spent more time (Massaro et al. 1974; Wiener et al. 1977), or similar amounts of time involved in maternal behavior relative to control dams (Crnic 1976). Konig (1989) reported that lactating house mice, *Mus musculus*, spent similar amounts of time nursing and licking their pups independent of whether they were food restricted or not. However, food-restricted lactating mice spent

less time involved in maternal behavior relative to those of control dams; the former dams also reduced the size of their litter (Marsteller and Lynch 1987). Perrigo (1987, 1990) discovered that lactating house mice facing increased energy demands reduced the size of their litters by cannibalizing pups during the first 12 days, resulting in a greater mass at weaning for their surviving pups. Conversely, white-footed mice, *Peromyscus leucopus,* did not reduce the size of their litter.

A common feature of many of these studies in rats and mice was that dams were food restricted during all lactation; this represents an extreme condition and females facing such low availability may not mate and would not get pregnant (Sabau and Ferkin 2012). However, many rodents could face acute food restriction at different times during lactation (Bronson 1989). Thus, females kept on a restricted diet during early lactation may not display the same changes in maternal behavior as females restricted during middle or late lactation. For example, during early lactation dams spent much of their time in the nest nursing and licking their pups (Champagne et al. 2003; Kristal 2009; Rosenblatt and Lehrman 1963). The amount of time dams lick their pups affects their pups' ability as adults to form affiliations with same-sex conspecifics, potential mates, their exploratory behavior, and how much time they spend licking their own offspring (Champagne et al. 2003; Francis et al 1999; Moore 1984, 1992). Food-restricted dams may have to increase the amount of time they have to forage and have less time to spend involved licking their young and displaying other maternal behaviors. During middle lactation, pups open their eyes, begin eating solid food, and increase their locomotor activity (McGuire and Novak 1984; Rosenblatt and Lehrman 1963; Smotherman and Bell 1980; Solomon 1993). Since food-restricted females may spend less time involved in

maternal behavior, their pups may grow more slowly, have lower body mass, and may be less likely to move around and be active as adults relative to pups raised by dams that were not food restricted during middle lactation. During late lactation pups spend much of the time exploring the area within and outside their mother's territory, usually near their mother (Hayes and Solomon 2006, 2007; Kristal 2009; Rosenblatt and Lehrman1963). Food-restricted female voles may spend less time near their pups, which may result in weanlings that are less inclined to interact with conspecifics or investigate novel areas. Thus, the maternal behavior of dams and the growth and behavior of their pups may be affected by when food restriction is initiated during lactation.

Female meadow voles, *Microtus pennsylvanicus,* may be susceptible to food restriction, especially during lactation. Female meadow voles are income breeders; they do not cache food (Batzli 1985). During the breeding season, female voles live in mutually exclusive territories that vary in the quality and amount of forage (Bergeron and Jodoin 1987, 1989; Madison 1980). Thus, the amount of food that is currently available to a lactating female may affect the amount of time that she can dedicate to maternal behavior. Moreover, female voles can produce multiple litters in their short life span (Keller 1985; Tamarin et al. 1984). Food-restricted dams may reduce their maternal investment in their current litter through an adjustment in litter size and/or a reduction in maternal behavior to maintain sufficient energy reserves for future litters (Trivers 1972).

In this study, we determined if the maternal behavior of lactating, food-restricted female meadow voles differs from that of lactating, female voles that had continuous access to food. We hypothesized that the amount of time female voles provided maternal behavior would differ if they were deprived of food during early, middle, or late in

lactation. We did so, because the energy demands differ, the growth and development of pups change, and the frequency of the behaviors displayed by females towards their pups change during early, middle, and late lactation (McGuire and Novak 1984; Hayes and Solomon 2006, 2007). We considered lactation days 1-7, 8-14, and 15-21 as early, middle, and late lactation, respectively.

MATERIALS AND METHODS

Animals.

We used meadow voles that were 3rd–4th generation descendants of free-living voles captured in Pennsylvania, Kentucky, New York, and Ohio. The voles were born and raised under a long photoperiod (14:10 h, L:D, lights on at 0700 h CST). Voles were weaned at 21 days of age, housed with littermates until 34 days of age, and thereafter housed singly in clear plastic cages (27 x 16.5 x 12.5 cm, l x w x h), until they were 5-9 months and paired with a male for 3 days. Cages contained bedding, water, and food (Harlan Teklad Rodent Diet, #8640, Madison, Wisconsin). All the female voles used in this study were sexually experienced, having delivered and weaned a litter. Males used in this experiment were 5-9 month-old and sexually experienced, having sired 1 litter. At the start of the experiment, all male and female voles had been housed singly for 4 weeks. Female meadow voles do not undergo regular estrus cycles (Keller 1985); they are induced ovulators that will readily mate with males when housed together under a long photoperiod (Meek and Lee 1993; Milligan 1982). We followed Animal Care Protocol 0647, which was approved by the IACUC at The University of Memphis and the ASM guidelines for research involving live mammals (Sikes et al. 2011).

Procedure.

We paired sexually experienced males (n = 53) and females (n = 53) and allowed them to mate in a breeding cage (30 cm x 24 cm x 14.5 cm, l x w x h). The male and female pairs had continuous access to food and water. After 3 days, the males were removed from the breeding cage and returned to their home cages. We began checking litters 3 times daily (0800, 1500, and 2100 h) 20 days after the females were paired with a male. Forty-four of the 53 females that delivered pups between 21 and 24 days later were randomly assigned to one of the following groups: 1) dams that had continuous access to food, the control group, 2) dams that were 30% food restricted days 1-7 of lactation (FR 1-7), 3) dams that were 30% food restricted days 8-14 of lactation (FR 8-14), and 4) dams that were 30% food restricted day 15-21 of lactation (FR 15-21). There were 11 different females in each treatment group. It is important to note that by day 13, pups and dams were both eating solid food. Thus, pups in group FR 15-21 and to a much lesser extent group FR 8-14, also faced a 30% food restriction during lactation.

We selected a 30% food restriction because previous studies found that 15% food restriction was too mild and 60-80% food restriction too severe (Crnic 1980; Marsteller and Lynch 1987; Schneider and Wade 1989). To validate our food-restriction protocol, we conducted a pilot study using a 30% food restriction and found that all of our dams (n = 5) survived, did not cannibalize their young, and weaned their litters.

Calculating food restriction.

The dams were provided with 30 g of food daily at 1700 h starting on the first day of lactation. Twenty-four h later, we removed the dam and collected and weighed any food that remained in the cage-lid hoppers and on the floor of the cage to determine food intake (Ohaus GT4000 Automatic Balance, Florham Park, New Jersey). We calculated the food intake of control females every day of lactation. We then gave the females in the food-restricted group 30% reduction of the food intake of control females from the previous day of lactation.

Determining body mass.

We recorded the body mass of females between days 7-10 and 17- 20 of gestation, and on days 1, 3, 8, 12, 15, 18, and 21 of lactation. All masses were recorded at 1700 h, the time that we provided the dams with food for the next day. We also recorded the mass of each pup within a litter at birth, the number of pups born into each litter, changes in litter size, and changes in each pup's mass every 3-5 days, from parturition until the pups were separated from their litter mates on day 34. After weaning, pups from all groups were fed ad libitum. We marked each pup with a distinctive number using black hair dye.

Maternal behaviors.

We recorded the maternal behaviors of each lactating female from day 1 of lactation until the pups were weaned on day 21. All dams and their pups were observed daily, at 0800 h and at 2200 h, to encompass the onset of the light and the dark phases of the light cycle, respectively. During the dark phase of the light cycle all observations of

maternal behavior were conducted under a red light. Each observation session was approximately 40 min long.

During each observation period, we recorded the total amount of time that a dam was a) nursing at least one pup, b) licking the body and/or anogenital area of a pup during nursing or outside of the nursing bout, c) constructing the nest or manipulating existing nesting material, and d) time spent within 2 cm or less of one or more of her pups. Similar behaviors have been measured in studies of maternal behavior in voles and other rodents (Ferkin 1987; Hayes and Solomon 2006, 2007; McGuire and Novak 1984). The maternal behavior of each dam was scored for 2 minutes, at 8-minute intervals, 4 times in a 40-minute time interval. Each dam was observed twice a day, for a total of 42 observations across the 21 days of lactation. We calculated the mean (± *SE*) time that the dams spent displaying these maternal behaviors during each lactation period. The observer was positioned 65 cm away from the transparent cage containing the dam and her pups. We used stopwatches and counting boxes to score maternal behaviors (Ferkin 1987).

Statistical analyses.

We used separate 2-way repeated measures analysis of variance (ANOVA) to determine whether significant differences existed in the body mass of the dams and body mass of the offspring in the different treatment groups across lactation. If statistically significant differences were revealed, we conducted 1-way repeated measures ANOVA's followed by Holm-Sidák post hoc pairwise comparisons to determine the significant treatment effects. We used the average mass of each litter to determine if the mean body

mass of pups differed between treatment groups. In addition, we used separate 2-way ANOVA's (dams in the treatment and control group × lactation phase) to determine whether significant differences and interactions existed in the time spent in each of the maternal behaviors by dams in the different treatment groups across lactation. If statistically significant differences were revealed, we conducted 1-way ANOVAs followed by Holm-Sidák post hoc pairwise comparisons to determine the significant treatment effects. Significant differences were accepted at $P < 0.05$ for all statistical tests.

RESULTS

Dam's body mass.

Control females, FR 1-7, FR 8-14, and FR 15-21 females lost mass throughout lactation ($F_{6, 240} = 18.20$, $P < 0.001$). There was also a significant interaction between restriction group and time ($F_{18, 240} = 6.18$, $P < 0.001$). FR 1-7 and FR 8-14 dams had a more severe decrease in body mass compared to FR 15-21 dams and control dams (Holm Sidák, both comparisons, $P < 0.05$; Fig. 1).

At the beginning of lactation dams had similar body mass; there was no significant difference between treatment groups on day 1 ($P > 0.05$). FR 1-7 dams weighed less than all the other groups on day 8 of lactation ($P < 0.05$). FR 1-7 dams lost 17.6% of their body mass by day 8 of lactation whereas control dams maintained their body mass. FR 8-14 dams weighed less than control dams during middle lactation on day 12 ($P < 0.05$; Fig. 1). FR 8-14 dams lost 15.78% of body mass by day 12 of lactation whereas control dams maintained their body mass. By day 15 control females lost 3.1 %

of their body mass. There was no significant difference ($P > 0.05$) in body mass of FR 15-21 dams and that of control dams at any time during lactation. During late lactation, control dams lost 8.9 % of their body mass whereas FR 15-21 dams lost 11% of their body mass. On day 21 no significant differences existed in the body mass of females across treatment groups ($P > 0.05$; Fig. 1).

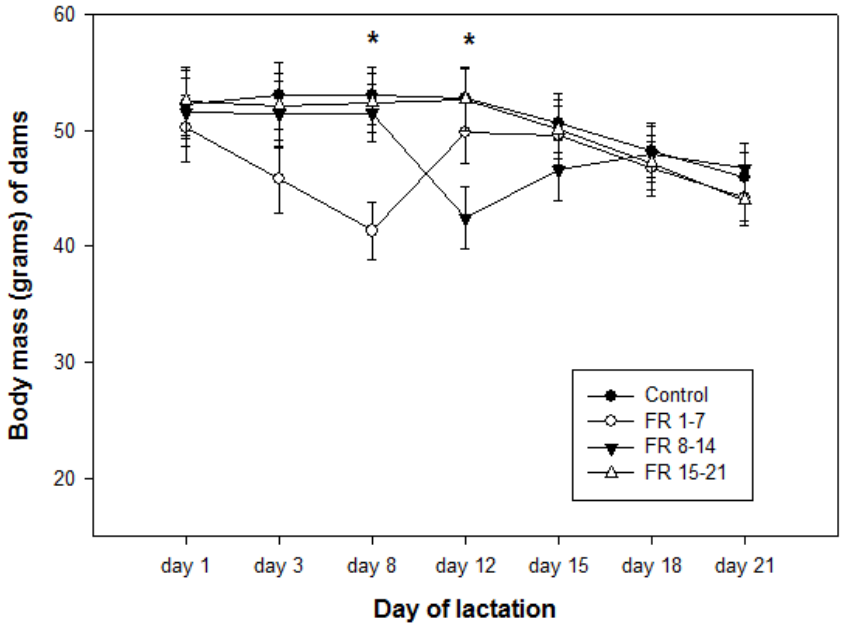

Figure 1. Mean ± *SEM* body mass (g) of control, FR 1-7 dams, FR 8-14 dams, and FR 15-21 dams across lactation (days 1-21). Asterisk (*) indicates significant difference at $P < 0.05$.

Survival of pups.

We found no difference among the dams in the number of offspring that survived to weaning on day 21. The mean litter size at weaning for the dams was similar (4.2 ± 0.49 pups per litter) and did not differ between groups ($F_{3, 43} = 0.89$, $P = 0.45$).

Nursing the pups.

The amount of time that dams spent nursing throughout the lactation period differed with respect to whether the dams were in early, middle, or late lactation ($F_{2, 72}= 182.43$; $P < 0.001$), and treatment ($F_{3, 72}= 8.16$, $P < 0.001$). There was a significant interaction between time interval and treatment ($F_{6, 72}= 2.83$, $P = 0.016$). To understand this interaction, we conducted separate 1-way ANOVAs for each time interval. There was a significant difference between groups in the time spent nursing during the first 7 days of lactation ($F_{3, 24} = 4.03$, $P = 0.019$; Fig. 2). The amount of time that females nursed their pups was lower for FR 1-7 and FR 8-14 dams than it was for the FR15-21 and control dams ($P < 0.05$; Fig. 2a). During mid-lactation there was a significant difference among groups in the amount of time dams spent nursing their young ($F_{3, 24} = 8.82$, $P < 0.001$). FR 8-14 dams spent less time nursing their young compared to dams in the other groups ($P < 0.05$, each comparison; Fig. 2b). During late lactation, there was no difference among the groups in the amount of time dams spent nursing their young ($F_{3, 24} = 1.51$, $P = 0.23$; Fig. 2c).

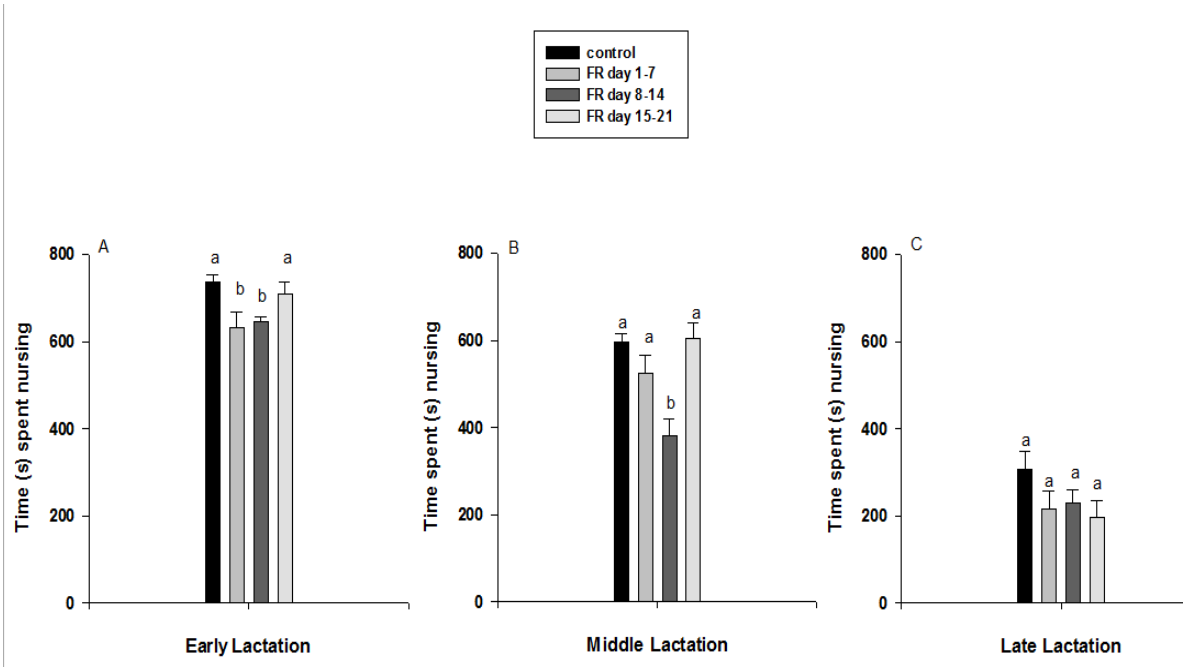

Figure 2. Mean ± *SEM* amount of time (s) that control, FR 1-7 dams, FR 8-14 dams, and FR 15-21 dams spent nursing their pups during a) days 1-7 of lactation, b) days 8 -14 of lactation , and) days 15-21 of lactation. Histograms capped with different letters denote significant differences at $P < 0.05$.

Licking the pups.

The amount of time that dams spent licking their pups differed across time intervals ($F_{2, 72}$= 96.81, $P < 0.001$) and was affected by whether females were food restricted and when the food restriction occurred ($F_{3, 72}$ = 21.21, P < 0.001). A significant interaction existed between the period of lactation and the treatment for females ($F_{6, 72}$ = 9.25, $P < 0.001$). As such, we once again used 1-way ANOVA to understand this interaction. Dams differed in the amount of time they spent licking their pups during the

first 7 days of lactation ($F_{3, 24} = 25.53$, $P < 0.001$). FR 1-7 dams spent significantly less time licking the pups compared to that of dams in the other groups (Holm Sidák, each comparison $P < 0.05$; Fig. 3a). The time dams spent licking their pups differed across middle lactation ($F_{3, 24} = 7.68$, $P < 0.001$). FR 8-14 dams spent significantly less time licking their pups compared to control females ($P < 0.05$; Fig. 3b). During mid-lactation, FR 1-7 dams continued to spend less time licking their pups than did control females ($P < 0.05$; Fig. 3b). During late lactation, there was no difference among the groups in the amount of time that females licked their young ($F_{3, 24} = 1.46$, $P = 0.24$).

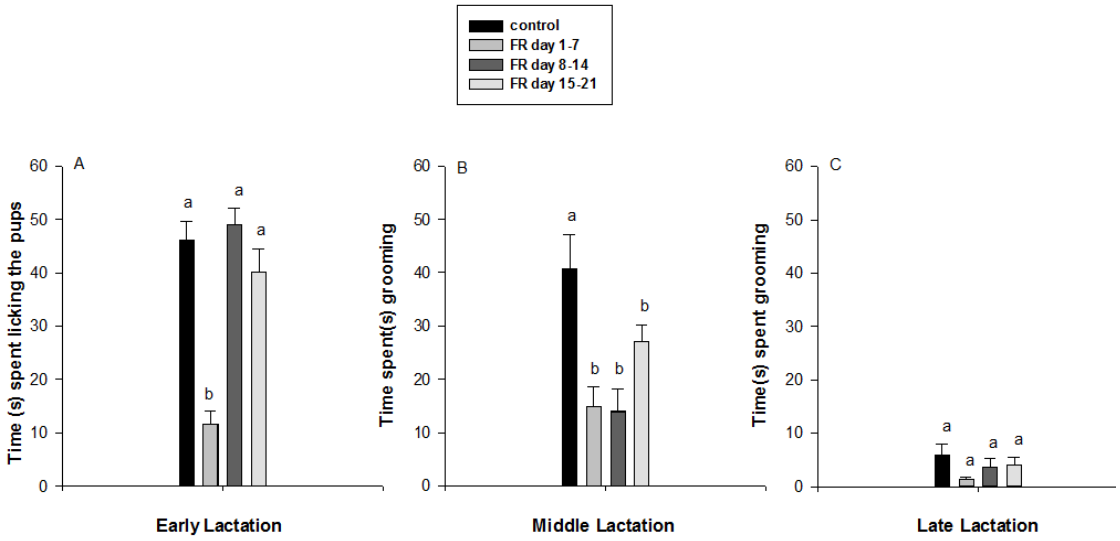

Figure 3.—Mean ± *SEM* amount of time (s) that control, FR 1-7 dams, FR 8-14 dams, and FR 15-21 dams spent licking their pups during a) days 1-7 of lactation, b) days 8 -14 of lactation , and c) days 15-21 of lactation. Histograms capped with different letters denote significant differences at $P < 0.05$.

Time spent with young.

The amount of time that dams spent with their young outside of the nest differed between treatment ($F_{3, 72} = 6.94$, $P < 0.001$) and by period of food restriction ($F_{2, 72} = 93.15$, $P < 0.001$). There was a significant interaction between lactation interval and treatment ($F_{6, 72} = 5.23$, $P < 0.001$). Dams in the FR groups and those in the control group spent similar amounts of time with their young outside the nest during early ($F_{3, 24} = 0.104$, $P = 0.95$) and middle lactation ($F_{3, 24} = 0.91$, $P = 0.45$). However, differences existed in the amount of time that dams spent with their litters during late lactation ($F_{3, 24} = 7.07$, $P < 0.001$). At this time, FR 15-21 and FR 8-14 dams spent less time with their young than did control dams (Holm Sidák, $P < 0.05$, each comparison). Also, dams FR 8-

14 spent less time in contact with pups compared with dams FR day 1-7 ($P < 0.05$; Fig. 4).

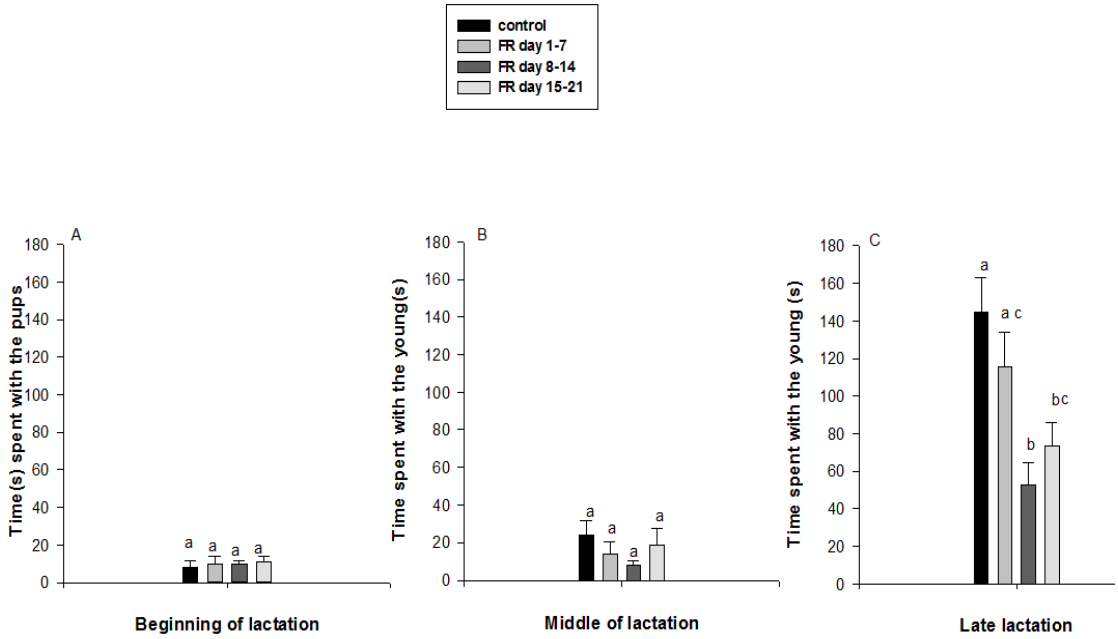

Figure 4.—Mean ± *SEM* amount of time (s) that control, FR 1-7 dams, FR 8-14 dams, and FR 15-21 dams spent outside the nest with their pups (not nursing) during a) days 1-7 of lactation, b) days 8 -14 of lactation, and c) days 15-21 of lactation. Histograms capped with different letters denote significant differences at $P < 0.05$.

Nest maintenance.

The amount of time that dams spent involved in nest maintenance varied across early, middle, and late lactation ($F_{2, 72}= 7.98$, $P < 0.0001$). There was no difference between treatment groups ($F_{3, 72}= 0.53$, $P = 0.65$). There was also no significant interaction between the variables ($F_{6, 72}= 1.05$, $P = 0.40$), indicating that the decrease in time spent for nest maintenance was independent of whether or not the dam was food

restricted during lactation. During early lactation, all dams spent similar amounts of time involved in nest maintenance ($P < 0.05$); by mid lactation, these dams ceased nest maintenance.

Pup growth.

Maternal food restriction affected the body mass of pups ($F_{6, 240}= 893.6$, $P < 0.001$) and food restriction interval affected the pup's mass ($F_{3, 40}= 11.10$, $P < 0.001$). A significant interaction existed between treatment and interval ($F_{18, 240}= 10.20$, $P < 0.001$). After day 7, control pups weighed significantly more than pups raised by FR 1-7 dams and those raised by FR 8-14 dams (Holm Sidák, $P < 0.05$; Fig. 5). With the exception of day 21, no significant difference was found in the body mass of pups reared by FR 15-21 dams compared with controls (Fig. 5).

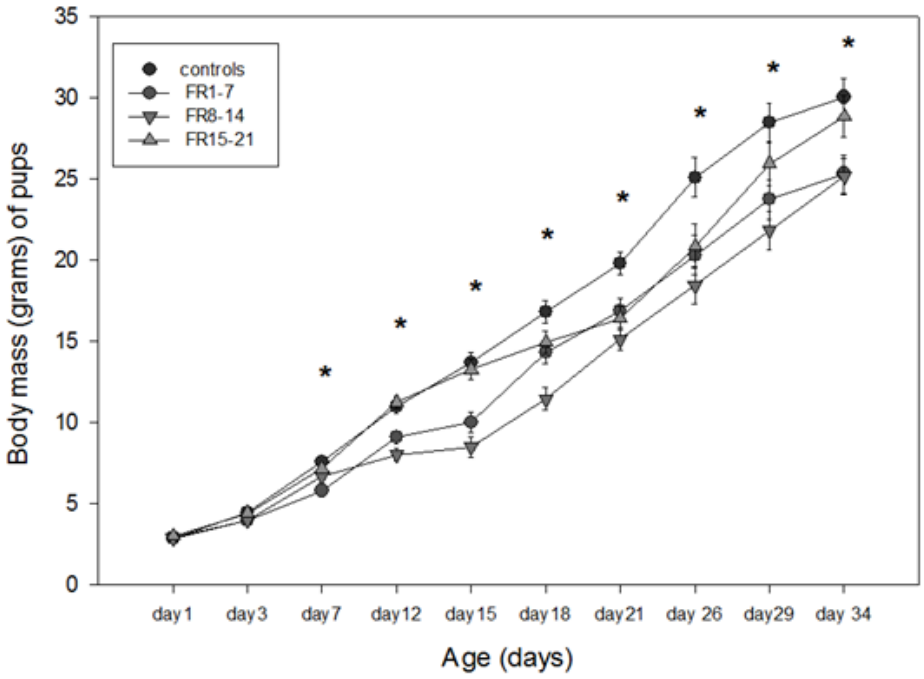

Figure 5.—Mean ± *SEM* body mass (g), from birth to day 34 of pups whose mothers were fed ad libitum, and those whose mothers were FR day 1-7, FR day 8-14, or FR day 15-21 during lactation. Asterisk (*) indicates significant differences at $P < 0.05$.

DISCUSSION

Our data support the hypothesis that the amount of time female voles provided maternal behavior would differ if they were deprived of food during early, middle, or late in lactation. We found that dams that were food restricted during early or middle lactation spent less time nursing their pups than did control dams. Pups whose mothers were restricted during early and middle lactation show impaired growth/mass at the time of weaning and did not show compensatory growth later. Pups whose mothers were on food restriction during late lactation were eating solid food by day 14. Thus, these pups may have also been food restricted, thereby contributing to their low body mass on day 21, at

weaning. Several studies have shown that weaning mass is positively correlated with overwintering survival and time to puberty (Beacham 1980; Dark et al. 1983; Desy and Thompson 1983). Lower-mass weanlings have fewer mating opportunities and lower reproductive success as adults relative to heavier-mass weanlings (Koskela 1998; Oksanen et al. 1999, 2002; Parker and Pizzari 2010; Wauters and Dhondt 1989). Our results support the speculation that changes in maternal behavior during the first 14 days of life could have long-term fitness consequences for voles after they are weaned.

We also discovered that FR 1-7 and 8-14 dams spent less time licking their pup compared to that of control dams. Similarly, lactating rats that were food restricted to 50% of the intake of food of control females during the first 10 days of lactation spent less time licking their young than did control dams (Smart 1976; Smart and Preece 1973). Reductions in maternal licking may affect a dam's fluid balance (Friedman et al. 1981; Gubernick and Alberts 1983), causing a reduction in her body mass as well as the growth, development, and behavior of her pups (Levy et al. 2004; Moore 1984, 1992; Schanberg and Field 1987). For example, rat pups that were licked less often displayed deficits in cognition, learning, and memory, as well as forming social affiliations, and mating once they became adults (e.g., Caldji et al. 1998; Moore 1984, 1992; Schanberg et al. 1984). More importantly, the amount of time that dams lick their pups affects the amount of time their daughters will lick their own offspring (Champagne et al. 2003; Francis et al. 1999). This in turn may influence their pups' attractiveness and response to opposite-sex conspecifics as adults. Changes in these aspects of the phenotype of voles may be sufficient to affect their mating and reproductive success (Berteaux et al. 1999; Boonstra et al. 1993), which could influence population demography (Tamarin et al. 1984).

Food-restricted female and control female meadow vole had weaned litters that were similar in size. However, pups reared by food-restricted females had lower mass at weaning compared to that of pups reared by control females. Likewise, mice dams that were food restricted to 60% and 80% of the intake of control dams, weaned pups that had lower body mass at weaning than control pups (Konig 1989; Marsteller and Lynch 1987). Our findings suggest that the lower mass at weaning of meadow vole pups may have been the result of receiving less maternal care from food-restricted females on days 1-7 or days 8-15 of lactation. However, we cannot rule out the possibility that maternal malnourishment may have affected the pups' ability to elicit maternal stimulation. Massaro et al. (1974) reported that rat pups reared by dams malnourished during lactation showed deficits in behaviors such as movement from the nesting area, feeding and drinking, rearing and climbing. It is also possible that these deficits in the pups' behavior were due to the fact that milk production or composition of a dam's milk is altered by nutritional state of the mother (e.g., Crnic and Chase 1978; Kliewer and Rasmussen 1987; McGuire et al.1995; Muller and Cox 1946; Rogowitz 1996).

We found that dams that were food restricted between days 1-7 and days 8-14 of lactation lost significantly more mass than did control dams. Since dams are under high energy demands during lactation (Gittleman and Thomson 1988; Migula 1969; Rauw et al. 2003), a severe decrease in body mass may indicate that dams may have reached a physiological limit and are unable to maintain their energy balance. However, our dams did not abandon their litter or cannibalize their pups to reduce energy costs as did female house mice (Perrigo 1987, 1990). Instead, food-restricted female voles spent less time relative to control females displaying maternal behavior towards their pups. Thus, our

findings are consistent with the speculation that food-restricted, lactating female meadow voles display a trade-off between investing in current offspring and surviving to produce future litters (i.e., Therrien et al. 2007, 2008; Trivers 1972).

At present, there seems to be no general pattern to explain the effects of food restriction on the maternal behavior of female rodents. This may be due to studies using different methods, diets, and degrees of food restriction (Crinic 1976; Smart 1976; Wiener et al. 1977; Konig 1989). Lack of a pattern could also be due due to researchers using different species or strains of rodents that have different reproductive physiology, social and mating systems, timing of breeding, and litter sizes. For example, rats and mice are omnivores, opportunistic breeders, have repeated and frequent interactions with conspecifics and females undergo estrous cycles, are spontaneous ovulators, and have relatively large litter sizes (Eisenberg 1967; Bronson 1989; Dewsbury 1990). In contrast, meadow voles are herbivores; are seasonal breeders; have few repeated and frequent interactions with conspecifics and females do not undergo estrous cycles, are induced ovulators, and have smaller litter sizes (Dewsbury 1990; Eisenberg 1967; Keller 1985, Milligan 1982). In many rodents, litter size is negatively correlated with maternal behavior (McGuire and Bemis 2007). However, maternal behavior was independent of litter size in Guenther's vole, *Microtus socialis guentheri*; a species in which individuals have repeated and frequent interactions with conspecifics and are herbivores (Libhaber and Eilam 2004). A more promising approach to determining a pattern of maternal behavior may be to compare capital breeding and income breeding females. Capital breeding females use previous food stores to augment periods of low food availability, whereas income breeding females consume food that is currently available (Houston et

al. 2007). Female meadow voles may be considered income breeders and deer mice may be considered capital breeders (Vander Wall et al. 2001); however food-restricted females in both species attempted to rear their litters to weaning without culling them (this study; Perrigo 1987, 1990). Nevertheless, more research may need to focus on comparisons between capital and income breeders in a variety of species.

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CHAPTER 4

Maternal food-restriction during lactation affects body weight and sexual behavior of male offspring in meadow voles (*Microtus pennsylvanicus*)

INTRODUCTION

Individual differences in phenotype have received much attention in the literature, however, little is known about the proximate causes of this variation (Forstmeier et al. 2004). Recently, maternal effects have been widely recognized as a mechanism for phenotypic responses to environmental heterogeneity (Bernardo 1996; Mousseau & Fox 1998a). Thus, maternal effects may influence an offspring's fitness (Mousseau & Fox 1998a, b; Gendreau et al. 2005). Several studies have shown that the nutritional status of dams during pregnancy and lactation can alter the phenotype of their offspring in many species (Passos et al. 2000; Kerr et al. 2007). Maternal malnutrition as a result of food deprivation or restriction not only affects birth weight or growth and development of offspring (Woodall et al. 1996; Teixeira et al. 2002), but also the physiology and behavior of offspring. Among mice and rat-like hamsters, food restriction during pregnancy resulted in dams producing sons that had lower body weight and lower social status relative to those of dams that were not food restricted (Meikle & Thornton 1995; Meikle et al. 1995; Liang et al. 2004). Maternal food restriction or a diet low in protein can also affect the reproductive physiology of male offspring, usually by delaying the onset of puberty (Engelbregt et al. 2000; Da Silva et al. 2001; Leonhardt et al. 2003; Guzman et al. 2006).

Most interesting, however, is that maternal food-restriction can have persistent effects on the reproduction of male offspring when they reach adulthood. Male rat-like

hamster (*Cricetulus triton*) dams that were food restricted during gestation had smaller gonads and lower gonadal steroid hormone titers relative to those of offspring of control dams (Liang et al. 2004). In rats (*Rattus norvegicus*), maternal protein restriction during pregnancy and lactation decreased sperm count and fertility in male offspring (Zambrano et al. 2005). Food restriction during pregnancy resulted in dams producing sons that had lower body weight and lower social status relative to those of dams that were not food restricted in mice (*Mus musculus*) (Meikle & Thornton 1995; Meikle et al. 1995) and rat-like hamsters; Liang et al. 2004). A general consensus of the literature suggests that food restriction of dams during pregnancy and/or lactation negatively impacts the morphology, physiology, and behavior of their sons, and that these long-term effects are expressed in offspring in the next generation.

A common feature of these and similar studies on the behavior of offspring raised by nutritionally-challenged females was that food availability was reduced during the entire gestation and/or lactation period. This represents an extreme condition and females facing such low food availability may not mate or become pregnant (Sabau & Ferkin 2013a). Many rodents, however, could experience acute food restriction at different times during lactation (Batzli 1985; Bronson 1989). For example, female meadow voles, *Microtus pennsylvanicus*, become relatively sedentary prior to parturition and during the first days of lactation (Madison 1980, 1985; Keller 1985; Sabau & Ferkin 2013a) Meadow vole dams would be limited to the forage that is available in their territory and susceptible to food restriction during the three weeks it takes to wean a litter (Batzli 1985; Sabau & Ferkin 2013b).

Our recent work has shown that food restriction during early and middle lactation (but not during late lactation) reduces the amount of time that dams nurse their pups relative to that of control dams (Sabau & Ferkin 2013b). In that study, males reared by dams were food restricted during early lactation, which was considered days 1-7 of lactation (FR 1-7), middle lactation, which was considered days 8-14 of lactation (FR 8-14), and late lactation, which was considered days 15-21 of lactation (Sabau & Ferkin 2013b). Sabau and Ferkin (2013b) found that FR 1-7 males and FR 8-14 males had a lower body weight at weaning compared to FR 15-21 males and males reared by control dams. However, the body weight of FR 15-21 males and control males was similar (Sabau & Ferkin 2013b). In addition, they found that meadow vole dams that were food restricted during middle lactation spent less time licking their pups compared to control dams and dams that were food restricted during late lactation (Sabau & Ferkin 2013b). Rodents dams that spend less time licking their pups produce adults that may not be able to interact successfully with same- and opposite-sex conspecifics (Moore 1982, 1993; Champagne et al. 2003). In addition, maternal food restriction during early and middle lactation may also affect the reproductive system of male rodents, which becomes fully developed by 10 days of age (Larsson et al. 1974), which could impact on their sexual behavior in adulthood (Govic et al. 2008).

The goal of the present study was to test the hypothesis that male offspring of meadow vole dams that were food restricted for different periods of time during lactation show persistent, negative effects on their sexual behavior as adults. The results for female offspring are presented in another paper. We predicted that these effects would be more pronounced in the FR 8-14 males than in the FR 15-21 and the control males due to the

fact that mid lactation is the most demanding period of lactation for dams and pups go through accelerated growth and development during this period. We tested this hypothesis and prediction by measuring the three components of sexual behavior: attractivity, proceptivity/interest in the opposite sex, and receptivity (Beach 1976). Attractivity, for meadow voles, like many other terrestrial mammals, refers to the attractiveness of one's odors and scent marks to opposite-sex conspecifics (Pierce et al. 2005). Proceptivity includes the behaviors displayed by females and by males to show interest in and to facilitate interactions with opposite-sex conspecifics, such as investigating the scent marks of potential mates (Johnston 1979; Pierce et al. 2005; Hobbs & Ferkin 2012). Attractivity and proceptivity establish communication between potential mates, and allow them to coordinate behaviors that facilitate or inhibit direct interactions (Beach 1976; Stopka & Macdonald 1998; Ferkin 2011). Receptivity is characterized by a male's or a female's willingness to mate (Beach, 1976; Pierce et al. 2005). In females, lordosis is an indicator of receptivity (Gray & Dewsbury 1975; Pierce et al. 2005; Sabau & Ferkin 2013a). In males, receptivity can be scored by counting the frequency and duration of mounts, intromissions, thrusts, and ejaculations.

METHODS

Animals

We used meadow voles that were 3rd - 4th generation descendants of free-living voles captured in New York, USA. The voles were born and raised under a long photoperiod (14:10 h, L: D, lights on at 0700h CST). Voles used in this study had been housed singly in clear plastic cages (27 x 16.5 x 12.5 cm, l x w x h) for 4 weeks prior to

the initiation of these studies. These voles had continuous access to water, food (Harlan Teklad Rodent Diet, #8640, Madison, WI, USA), and cotton nesting material. We followed Animal Care Protocol 0647, which was approved by the IACUC at The University of Memphis. We adhered to the 'Guidelines for the use of animals in research' as published in *Animal Behaviour* (1991, 41:183–186) and the laws of the country where the research was conducted.

Male voles used in this study were offspring of dams that were either food restricted during early, middle, and late lactation or not (control group) in a recent study by Sabau and Ferkin (2013b). These males were offspring of 44 different litters. In that study, Sabau and Ferkin (2013b) randomly assigned day-1 lactating female meadow voles to one of the four groups of 11 dams each. These four groups were comprised of dams that had continuous access to food throughout lactation (control), and dams that were provided with 70% of the daily intake of the control dams between day 1 and 7 (FR 1-7), between day 8 and14 (FR 8-14), and between day 15 and 21 of lactation (FR 15-21) (Sabau & Ferkin 2013a). Dams in the FR groups had continuous access to food on days when they were not food restricted. For example, dams in treatment group FR 8-14 were provided with 70% of the daily intake of control dams between days 8-14 of lactation but had continuous access to food between days 1-7 and between days 15-21 of lactation.

On day 22 of lactation, the pups from all four groups were weaned, housed with littermates in separate cages, and thereafter, provided with continuous access to food and water. No statistical differences existed in the number of male and female pups that were weaned per litter per treatment (4.2 ± 0.5 pups per litter; Sabau & Ferkin 2013b). When

the pups were 34 days-old, they were separated from littermates, and housed individually in clear polycarbonate cages (27 × 16.5 × 12.5 cm, l x w x h).

Body Weight of Male Offspring

Males from our three FR treatment groups and the control group (n = 12 males per group) were weighed to the nearest of 0.1 gram every 3-5 days when they were between 22 and 43 days old, and every 10 days thereafter until they were 98 days old.

Food Intake of Male Offspring

The food intake of male offspring from the treatment groups and control group was also monitored until they were 98 days old. Briefly, 30 grams of food was placed into the cage-lid hopper of each male. Twenty-four hours later, we removed the male from its cage and collected and weighed (Ohaus GT4000 Automatic Balance, Florham Park, NJ) any food that remained in the cage-lid hoppers and on the floor of the cage to determine his daily food intake.

Sexual Behaviors

We used 12 different males in each of the treatment groups (FR1-7, FR 8-14, and FR 15-21) and 18 different males in the control group in the sexual behavior component of the study. We began testing these males for sexual behavior (attractivity, proceptivity, and receptivity) when they were between 60 and 65 days-old. The male voles underwent a single attractivity, proceptivity, and receptivity test. We used males and females that were unfamiliar and unrelated to the voles with which they were tested. We did not use

more than two individuals from the same litter in any test to eliminate the potential for litter effects. We used a Latin Squares design to allow male voles to serve as scent donors in the attractivity tests and then as subjects in the proceptivity tests and receptivity tests (Pierce et al. 2005). That is, some males were subjects in the proceptivity tests first, some were first subjects in receptivity tests, and others were first used as donors in attractivity tests. A minimum of 3 days separated successive tests with the same vole.

Attractivity Component

Scent donors were 18 male voles from the control group and 12 males each from the FR 1-7, FR 8-14, and FR 15-21 groups. The males in the treatment groups were used as scent donors once; the males in the control groups were used as scent donors twice.

Subjects were 36 female voles that had continuous access to food and were 120-150 days of age, born and raised in long photoperiod, and housed singly for 30 days prior to testing. Females were randomly chosen from a pool of 68 sexually experienced voles that were unrelated to and unfamiliar with the males used in the attractivity tests. Female subjects were not currently pregnant or lactating, but were sexually experienced, having weaned a litter 30 days prior to testing. Female meadow voles do not undergo regular estrous cycles (Keller 1985) and are induced ovulators (Milligan 1982). Females used in this study will readily mate with males when housed together under a long photoperiod (Meek & Lee 1993; Pierce et al. 2005; delBarco-Trillo & Ferkin 2006).

Each female subject underwent a single 10-minute attractivity test that followed the procedures detailed elsewhere (Pierce et al. 2005; Sabau & Ferkin 2013a). Briefly, we recorded the amount of time in seconds that females spent investigating the anogenital

area scent marks of the following pairs of opposite-sex scent donors: 1) a FR 1-7 male versus a control male, 2) a FR 8-14 male versus a control male, and 3) a FR 15-21 male versus a control male.

Each female was exposed to the scent marks of a unique pair of male donors. The scent marks of these two males were placed on a clean, glass microscope slide (2.5 x 7.6 cm). Each slide was divided in three equal sections. Each section was 2.5 cm long. One end section contained a scent mark of a male donor that was reared by a food-restricted dam, while the other end section contained a scent mark of a male that was reared by a dam that had continuous access to food. The middle section contained no scent marks. Briefly, the anogenital area of a male donor was rubbed for approximately 5 seconds against the left- or right side of a clean slide. The position of the two scent marks was alternated on the left- or right-side of the slide for each test. After both scent marks had been placed on the slide, we suspended the slide on a clip and hook apparatus 1 cm above the substrate, against the wall opposite the female's nest. During the 10-minute attractivity test, we recorded continuously the amount of time that the female subject licked or sniffed (the subject's nose comes within approximately 1-2 cm) each scent mark and the clean section of the slide. The test began when the slide was placed into the cage of the female subject. Male voles were considered to produce more attractive scent marks if females spent significantly more time investigating their mark relative to that of another male (Pierce et al. 2005; Sabau & Ferkin 2013a).

Fresh scent marks from the anogenital area were obtained for each trial from each scent donor; anogenital area scent marks are deposited by voles in their runways (Ferkin et al. 2004). The experimenter wore disposable latex gloves to minimize human scent

transfer while handling all slides. The investigator recording the behaviors was blind to the treatment conditions of the male and female voles in the attractivity tests, as well as the proceptivity and receptivity tests listed below.

Proceptivity Component

We used a scent preference test that was similar to the attractivity test to determine whether maternal food-restriction affected the proceptive behavior of male voles. Scent donors were 48 females and 48 males that had been reared by dams that were not food restricted; the male and female scent donors were between 120-150 days of age. The subjects were 48 males from the FR diet-treatment groups (n = 12 males per treatment group) and 12 males from the control group. Each male subject was tested once with a unique pair of male and female scent donors.

We followed the methods for proceptivity testing developed by Pierce et al. (2005) and are similar to the attractivity test we described above, with one notable exception. In the proceptivity test, male subjects were exposed to a glass slide that contained the scent mark of a female scent donor and the scent mark of a male scent donor. During the 10-minute proceptivity tests, we recorded the amount of time male subjects investigated the end of the slide containing the scent mark of the male scent donor and the other end of the slide containing the scent mark of the female scent donor. The position of the scent marks of the male and female donors on the left- or right-side of the slide was alternated. Male subjects were considered to display proceptive behavior if they spent significantly more time investigating the odors of the female scent donor than those of the male scent donor (Pierce et al. 2005).

Receptivity Component

We used the same methods in testing receptivity as described by Pierce et al. (2005). Briefly, a male vole was paired with a sexually experienced, 120-150 day-old unfamiliar female vole in a clear, plastic cage (37 × 21 × 15 cm; l, w, h), containing hardwood shavings, nesting material and water. At the time of pairing the females were neither pregnant nor lactating. Male voles were 12 males from each of the FR treatment-groups and 12 males from the control group; one of the males in the control group died shortly after being paired.

We allowed each pair to interact for 4 hours; meadow voles typically mate within this 4-h period (delBarco-Trillo & Ferkin 2004; Vaughn et al. 2008, 2011). We recorded each 4-hour pairing with a Sony Handycam DCR-SR68. During playback, we scored whether or not the males mated. If so, we scored the number of ejaculations by each male and his latency to first ejaculation, which was the amount of time (seconds) that elapsed between the male's introduction into the female's cage and his first ejaculation, which are typical measures of male copulatory behavior (Dewsbury 1972, 1975; delBarco-Trillo & Ferkin 2004, 2006, 2007). Collectively, these measures are indicators of sexual responsiveness of males and receptivity (Pierce et al. 2005). The ejaculation is characterized by a short series of rapid thrusts and intromissions followed by a noticeable relaxation of the male meadow vole's pelvic region area and extension of his legs (Gray & Dewsbury 1975; delBarco-Trillo & Ferkin 2004, 2007; Vaughn et al. 2008).

We observed lordosis but did not measure it. Female meadow voles display lordosis after males mount them (delBarco-Trillo & Ferkin 2006). Thus, measures of mounting by males and lordosis by females are highly correlated (Dewsbury 1972, 1975).

Statistical Analyses

We used matched-paired t-tests to determine whether significant differences existed in the amount of time each subject spent investigating the scent marks of the two donors in the attractivity and proceptivity tests (Pierce et al. 2005). We used binomial tests to compare the number of males in each FR groups that mated (receptivity test) with those that mated in the control group. We used two separate one-way ANOVAs to determine if males paired with females differed among groups in their number of ejaculations and latency to first ejaculation. We used GLM repeated measures analysis of variance (ANOVA) to determine whether significant differences existed in the body weight and food intake of the offspring in the different treatment groups. Litter and not individual offspring was used as experimental unit in order to avoid pseudoreplication; we used the average mass of males in each litter to determine if the mean body weight of male pups differed between treatment groups. We conducted 1-way ANOVA's followed by Holm-Sidák post hoc pairwise comparisons to determine the significant treatment effects. Significant differences were accepted at $\alpha < 0.05$ for all statistical tests. We used SPSS 13.0 to analyze the data.

RESULTS

Body Weight of Male Offspring After Weaning

The body weight of male offspring was affected by food restriction during lactation ($F_{3, 35}$=2.7, $p < 0.001$) and whether food restriction occurred during early, middle or late lactation ($F_{3.1, 110.1}$= 359.5, $p < 0.001$). A significant interaction existed between these variables ($F_{9.4, 110.1}$= 2.0, $p < 0.05$). After weaning (day 21) and until day 48, control males weighed significantly more than males reared by FR 1-7 dams and those reared by FR 8-14 dams (Holm Sidák, $p < 0.05$; Fig. 1). With the exception of day 21, no significant difference was found in the body weight of males reared by FR 15-21 dams and those reared by dams that were not food restricted ($p > 0.05$; Fig. 1). Between 26 and 48 days of age, males reared by FR 1-7 and FR 8-14 dams had lower body weight than did males reared by FR 15-21 and control dams (Fig. 1), However, the body weight of males raised by FR dams and control dams was similar when they were between 49 and 98 days of age (Fig. 1). There was no difference among the dams in birth weights and number of offspring at weaning on day 21. The mean litter size at weaning was 4.2 + 0.69 pups and was similar for litters of control and FR dams ($F_{3,43} = 0.89$, $P = 0.45$).

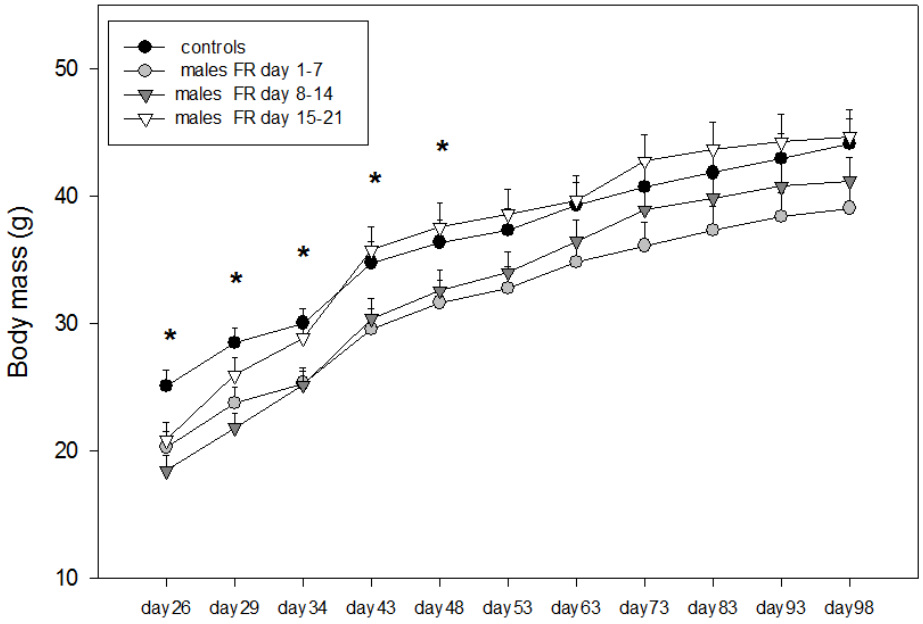

Figure 1. Mean ± SEM body weight (g), of FR 1-7 males, FR 8-14 males, FR 15-21 males, and control males. An asterisk (*) denotes significant differences between groups ($p < 0.05$).

Food Intake of Male Offspring After Weaning

All the male voles increased their food intake between days 34 and 98 ($F_{7, 238}$= 23.15, $p < 0.01$). However, there was no interaction between treatment (control or FR) whether food restriction occurred during early, middle or late lactation ($F_{21, 238}$= 1.48, p = 0.085). The amount of food consumed by the males was similar for FR males and control males between day 34 and day 98 ($F_{3, 34}$ =2.35, p > 0.05).

Attractivity

All female subjects investigated both scent marks, spending more time investigating the two scent marks of the two males than the middle portion of the slide. Maternal food-restriction during lactation affected the attractivity of scent marks of male offspring to female conspecifics (Fig. 2). Female voles spent significantly more time investigating the scent mark of control males than those of FR 8-14 males ($t_{11} = 3.51$, $p = 0.007$; Fig. 2). However, female voles spent similar amounts of time investigating the scent mark of a FR 1-7 male and that of a control male ($t_{11} = 0.78$, $p = 0.505$; Fig. 2) as well as the scent mark of a FR 15-21 male and that of a control male ($t_{11} = 1.82$, $p = 0.092$; Fig. 2).

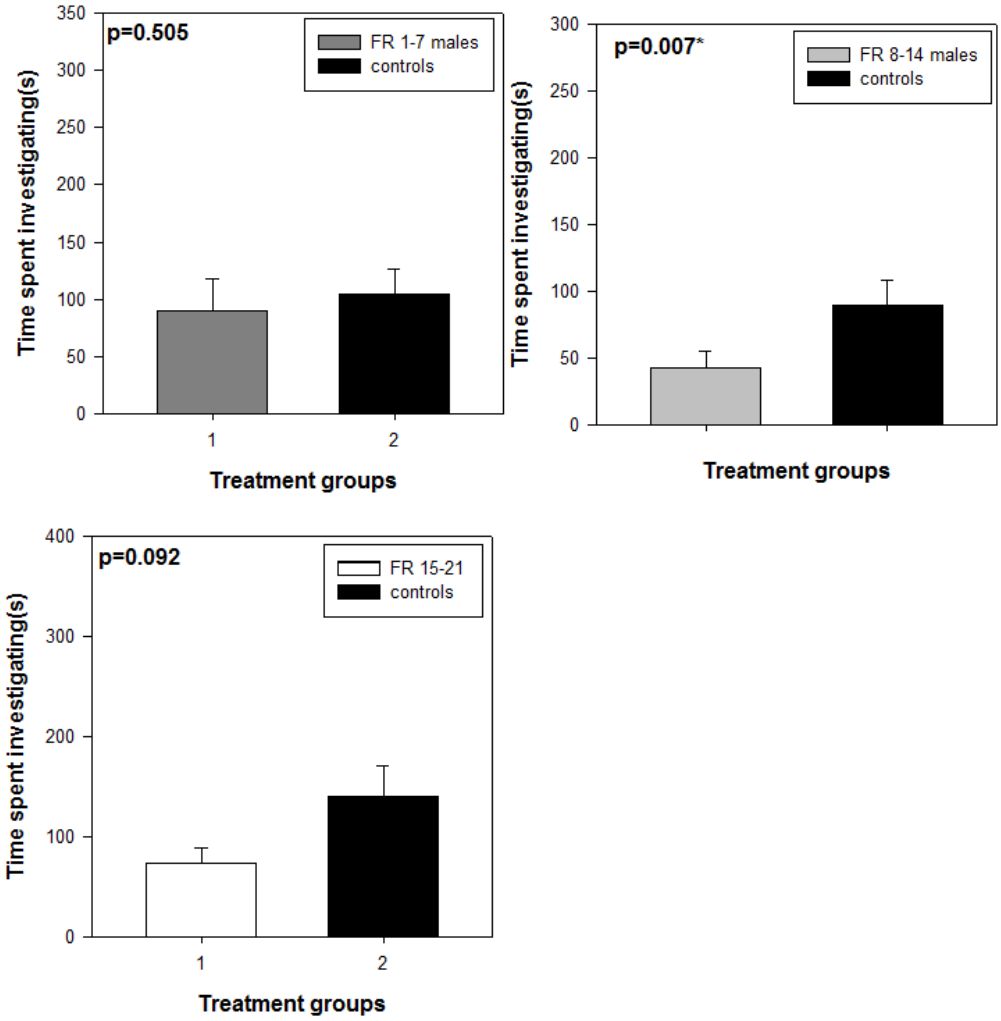

Figure 2. Mean ± SEM time (s) spent by female voles during a 10-minute test investigating the anogenital scent marks of in the following paired comparisons: a control male and a FR 1-7 male; a control male and a FR 9-14 male; and a control male and a FR 15-21 male. An asterisk (*) indicates significant differences between groups ($p < 0.05$).

Proceptivity

The preference for the scent marks of a female vole over the scent marks of a male vole was not affected by whether the males were reared by dams that were not food restricted or that were food restricted during early, middle, or late lactation (Fig. 3). FR 1-7 males ($t_{11} = 4.12$, $p = 0.001$), FR 8-14 males ($t_{11} = 2.83$, $p = 0.01$), FR 15-21 males ($t_{11} = 4.09$, $p = 0.002$) and control males ($t_{11} = 3.541$, $p = 0.005$) spent more time investigating the scent mark of female conspecifics compared to that of male conspecifics (Fig. 3).

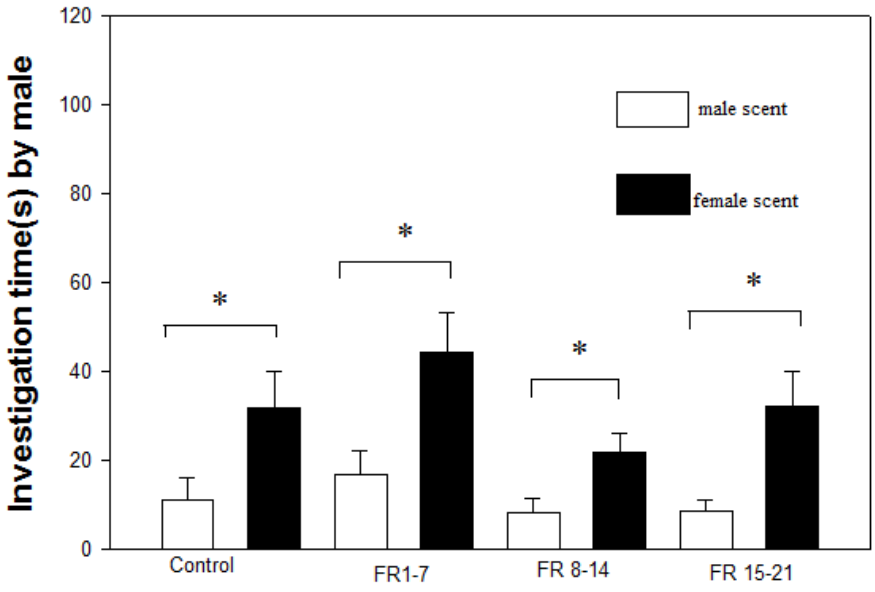

Figure 3. Mean ± SEM time (s) spent by control males, FR 1-7 males, FR 8-14 males, and FR 15-21 males during a 10-minute test investigating the anogenital scent marks of a male conspecific and a female conspecific. An asterisk (*) indicates significant differences between groups ($p < 0.05$).

Receptivity

We found that 9 of 12 FR 1-7 males, 4 of 12 FR 8-14 males, 8 of 12 FR 15-21, and 8 of 11 control males copulated with the females. FR 8-14 males had lower mating success relative to control (Binomial Critical Value test, $p = 0.006$; Fig. 4a). However, the mating success was similar for control males and FR 1-7 males (Binomial test, $p=0.555$) and controls and males FR 15-21(Binomial test, $p=0.445$). Thus, the number of males that copulated with a female vole was affected by whether the males were reared by dams that were food restricted during early, middle, or late lactation. There were also no significant differences in the latency to first mount ($F_{3, 25} = 0.7$, $p= 0.27$) and first ejaculation ($F_{3, 25}= 0.10$, $p= 0.85$; Fig. 4b) between FR males and control males or the number of ejaculations control and FR males had when paired with a female ($F_{3, 25} = 0.21$, $p= 0.93$; Fig. 4c).

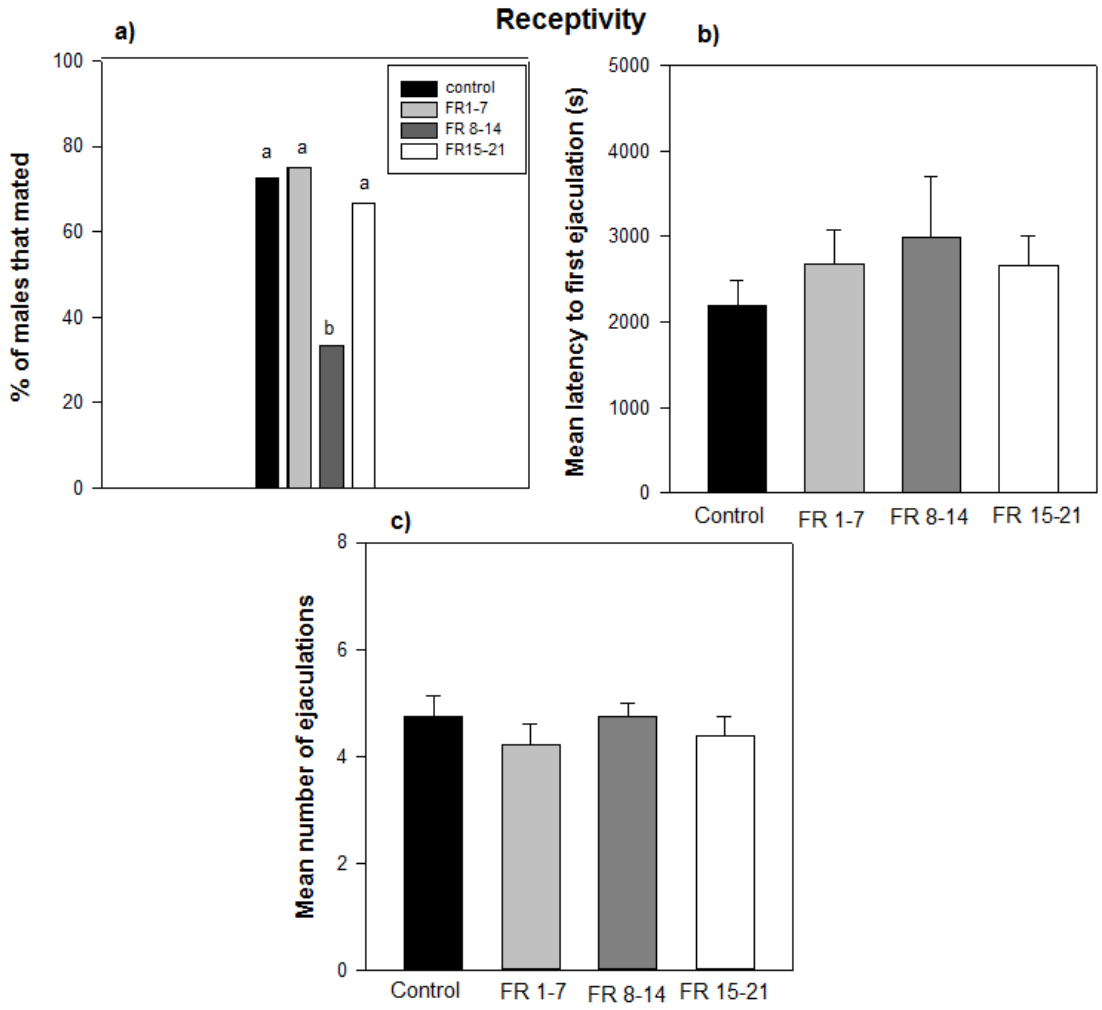

Figure 4a) Percentage of control males, FR 1-7 males, FR 8-14 males, and FR 15-21 males that mated when paired with a sexually receptive female vole for 4 hours. 4b) The latency to first ejaculation of of these control males, FR 1-7 males, FR 8-14 males, and FR 15-21 males. 4c) The mean ± SEM number of ejaculations of these control males and the FR 1-7, FR 8-14, and FR 15-21 males. In Figure 4a the histograms capped with different letters indicate significant differences between groups ($p < 0.05$). In Figure 4b and 4c there were no significant differences between groups ($p > 0.1$).

DISCUSSION

Food restriction of female meadow vole dams during middle lactation induced persistent, long-term negative effects on the attractivity component of the sexual behavior of their male offspring. FR 8-14 males produced anogenital marks that were not as attractive as those produced by control males and those of FR 1-7 males and FR 15-21 males. The scent marks of FR 1-7, FR 15-21 and control males were similar to one another in the attractiveness to female conspecifics. Estrus female house mice and rat like hamsters were also more attracted to the scent marks of male conspecifics reared by dams that were not food restricted during gestation than to those of males reared by dams that were food restricted (Meikle et al. 1995; Liang et al. 2004). Males that produce scent marks that are more attractive to females would be more likely to signal their presence in an area to potential mates. Our data suggest that compared to their counterparts, FR 8-14 male voles are less likely than control males, FR 1-7 males, and FR 15-21 males to compete for mates. It is not clear why maternal food-restriction during early or late lactation had no effect on the attractiveness of scent marks produced by males but food-restriction during middle lactation had a negative and persistent effect on the attractiveness of the scent marks of male meadows to female conspecifics. We suggest that this impairment is due to a deficit associated with major development of meadow vole during middle lactation. During middle lactation, pups open their eyes, spend less time nursing, and begin to eat solid food (McGuire & Novak 1984; Nadeau 1985). Thus, it is possibility that the FR 8-14 males were also food-restricted at a time when they were beginning to eat solid food and receiving less milk from their mothers (McGuire & Novak 1984), and this nutritional challenge was sufficient to trigger changes in the odor-

producing tissues of males. The decrease in the attractiveness of the scent marks of FR 8-14 males may reflect a tradeoff between developing the tissues that support growth and development, which will increase the pup's likelihood of surviving and those needed for production of sexually discriminable scent marks, which will increase the pup's likelihood of attracting a mate as an adult.

Food restriction during lactation did not affect a male vole's interest in female conspecifics. Male voles, independent of whether they were food restricted or not, spent more time investigating the scent mark of a male donor to that of a female donor. Similarly, Hobbs and Ferkin (2011) reported that food availability did not affect the scent marking and over-marking behavior of male meadow voles when they encountered the scent marks of female conspecifics. Male rodents show interest in particular females by scent marking, self-grooming or investigating their scent marks (Hobbs & Ferkin 2011, 2012). These proceptive-like behaviors facilitate further interactions with potential mates (Beach 1976; Stopka & Macdonald 1998). Our findings suggest that male meadow voles do not show reductions in the behaviors that indicate their interest in females. However, male voles that experienced nutritional challenges during lactation may reduce their likelihood of finding potential mates.

We discovered that only 33.3% of the FR 8-14 males copulated during the receptivity test, whereas 75% of the FR 1-7 males, 66.6% of the FR 15-21 males, and 72.7% of the control males copulated. The relatively low mating success of FR 8-14 male voles may be associated with the pronounced reduction the amount of care they received relative to controls in lactation. Sabau and Ferkin (2013b) found that FR 8-14 dams spent little time compared to control dams, and FR 15-21 dams nursing and licking their pups.

Similarly, lactating rats that were food restricted to 50% of the intake of food of control females during the first 10 days of lactation spent less time licking their young than did control dams (Smart & Preece 1973; Smart 1976). In rats, reductions in maternal licking caused offspring to become less likely to form social affiliations and mate (Moore 1984, 1992). We cannot rule out, the possibility that the amount of nutrition FR 8-14 males received was not sufficient to support the development of neuroendocrine substrates that mediate sexual receptivity. Although we are not aware of any studies that have examined the effects of maternal-food restriction during different periods of lactation on the receptivity of male offspring, our results are similar to most studies that showed dams that were food-restricted during gestation gave birth to sons with deficits in some aspect of their mating behavior and reproductive physiology (Larsson et al. 1974; Rhees & Fleming 1981; Zambrano et al. 2005). Our results do not follow the same pattern as other those reporting that maternal-food restriction increased aspects of the sexual behavior of male offspring among rats (Tonkiss et al. 1984; Govic et al. 2008) or had no effect on sexual behavior of male offspring among sheep (*Ovis aries*)(Rae et al. 2002).

The body weight of FR 1-7 and FR 8-14 males was lower than that of control males and FR 15-21 males at 48 days of age; puberty occurs around this time for male meadow voles (Nadeau 1985). This result was similar to that of other studies reporting that male offspring of mice and rats reared by dams that were under nutritional stress weighed less at weaning compared to male offspring that were reared by dams that were not under nutritional stress (Teixeira et al., 2002). The low body weight at puberty for FR 1-7 and FR 8-14 males may represent a tradeoff between size and survival as juveniles (Gendreau et al. 2005; Fairbanks & Hinde 2013; Holekamp et al. 2013). It is

also possible that the low body of FR 1-7 and FR 8-14 males at puberty were associated with deficits in their attractivity and their receptivity, respectively. The fact that by 98 days of age the FR 1-7 and FR 8-14 males weighed the same as FR 15-21 males and control males suggest the former voles have experienced some type of compensatory weight gain, but that this weight gain was not sufficient to reverse the negative effects of maternal-food restriction on aspects of their sexual behavior.

Why does food restriction during early or middle lactation affect components of the sexual behavior and body weight of male offspring? It is possible that maternal-food restriction, food restriction experienced by the pups, or changes in the amount of maternal care provided by the dams singly or together is sufficient to induce epigenetic effects that can trigger persistent, impairments on the sexual behavior and weight of male meadow voles. Several studies have shown that nutirional challenges during lactation may affect the phenotype of offspring (McGowan et al. 2011; Fairbanks & Hinde 2013), their sexual behavior and reproductive physiology (Forstmeier et al. 2004) and ability to form attachments with opposite-sex conspecifics (Francis et al. 1999; Cameron et al. 2008), which can transcend generations (Champagne et al. 2003). For a male meadow voles facing nutritional challenges during lactation could affect their survival (Sabau & Ferkin 2013b) as well as their lifetime mating and reproductive success (Larsson et al. 1974; Boonstra et al. 1993; Berteaux et al. 1999).

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CHAPTER 5

Body mass and attractivity of female offspring are negatively affected by food restriction of meadow vole (*Microtus pennsylvanicus*) dams during lactation

INRODUCTION

Many studies have discovered that an offspring's phenotype may be influenced by nutritional challenges faced by dams during pregnancy and lactation (Passos et al., 2000; Langley-Evans, 2001; Kerr et al., 2007). The majority of the studies have focused on male offspring. Male rat-like hamster (*Cricetulus triton*) offspring of dams that were food restricted during gestation had smaller gonads and lower gonadal steroid hormone titers relative to those of offspring of control dams (Liang et al., 2004). In rats (*Rattus norvegicus*), maternal protein restriction during pregnancy and lactation decreased sperm count and fertility in male offspring (Zambrano et al., 2005). Food restriction of meadow vole (*Microtus pennsylvanicus*) dams during the first and second week of lactation resulted in male offspring that produced odors that were less attractive to females and that copulated fewer times than male offspring of control dams (Sabau & Ferkin, unpublished data). Among rodents, food restriction during pregnancy resulted in dams producing sons that had lower body mass and lower social status relative to those of dams that were not food restricted (Meikle & Thornton, 1995; Meikle et al., 1995; Liang et al., 2004). A general consensus of the literature suggests that food restriction of dams during lactation negatively impacts the morphology, physiology, and behavior of their sons, and that these long-term effects are expressed in offspring in the next generation.

In male offspring in a variety of mammals, food restriction during lactation likely triggered epigenetic responses that induced changes in body mass, reproductive

physiology, and behavior which may then impact their mating and reproductive success (Mousseau & Fox, 1998a, b; Gendreau et al., 2005), and could potentially transcend generations (Waterland & Jirtle, 2003). It is not known if such effects occur in female offspring, but the literature offers some support for this speculation. For example, female rats that were licked less frequently by their mothers had lower levels of estrogen receptor alpha ERα gene expression than did offspring which were licked more frequently by their mothers (Cameron, 2011); as adults, these females were less able to form affiliations with potential mates, showed alterations in receptivity, and spent little time licking their own pups (Francis et al., 1999; Champagne et al., 2001, 2003; Cameron et al., 2008; Cameron, 2011). Thus, the maternal diet may somehow affect the amount of time that dams lick their offspring (Sabau & Ferkin, 2013b). Food restriction could affect some other behaviorally or physiological responses in dams that are somehow transferred to subsequent generations epigenetically (Francis et al., 1999; Cameron et al., 2008; McGowan et al., 2011). It is also possible that food restriction of dams during lactation could affect the physiological and behavioral responses of their female offspring which could affect their daughters and granddaughters mating opportunities with male conspecifics.

There have been few studies examining the effects of food restriction of dams on the physiology and behavior of their female offspring. Some studies reported that female offspring of mice and rat dams that were under nutritional stress during gestation or lactation had lower body mass at weaning (Rossiter, 1996; Teixeira et al., 2002). The daughters of food-restricted rodent dams reached puberty later than did offspring of control dams (Mousseau & Fox, 1998a, b; Gendreau et al., 2005). Studies by Price and

Coe (1999) found that female rhesus monkeys (*Macaca mulatta*) that were born at a lower body mass gave birth to daughters that had low body mass. These female monkeys had a greater delay to their first conception as well as higher rates of pre-term birth and neonatal mortality (Price et al., 1999). Hlinák and Franková (1983) found that 80 day-old female rats reared by malnourished dams showed no change in lordosis and proceptive behaviors such as hopping, darting, and presenting posture. In house mice (*Mus musculus*), maternal food deprivation during pregnancy had no effect on the proportion of daughters that produced litters, the timing of the first estrus, or the body mass at weaning of female offspring (Drickamer & Meikle, 1988; Meikle & Westberg, 2001). In rats, maternal protein restriction during pregnancy and lactation delayed puberty and increased reproductive cycle length in their female offspring (Guzman et al., 2006).

We will increase our understanding of the effects of food restriction of dams on the growth and behavior of female offspring by using female meadow voles as the focal species. Meadow voles make ideal subjects because food-restriction during lactation may be sufficient to induce epigenetic effects that alter the body mass and behavior of their female offspring. We offer three reasons to support this view. First, acute food restriction during pregnancy reduced the attractivity and receptivity of dams, their likelihood of entering postpartum estrus, and the amount of maternal care directed towards their offspring during lactation (Sabau & Ferkin 2013a, b). Second, for almost a month, during late pregnancy and through most of lactation, meadow vole dams become relatively sedentary and spend much of their time in their nests within their territory (Madison, 1981; McShea & Madison, 1989). The sires do not remain in the nest with a female and do not provision her with food (Madison, 1980; Batzli, 1985). Thus, late pregnant and

lactating dams are limited by the amount of forage that is in their territory (Getz, 1985). However, female territories differ in the quality and quantity of forage (grass and forbs); some females may inhabit a territory that is limited in quality and quantity of the forage. Flooding during the spring and drought during the summer could also affect the amount of food that females have in their territory (Batzli, 1985; Bergeron et al., 1990). Consequently, many female voles could experience acute food restriction at some time during lactation (Lindroth & Batzli, 1984; Batzli, 1985), a period of high energetic cost to the mother (Migula, 1969). Lastly, Sabau and Ferkin (unpublished data) have recently discovered that meadow vole dams that were food restricted during lactation weaned male offspring with persistent, adverse effects on the growth and sexual behavior. These male offspring had lower body mass at weaning and as young adults compared to male offspring of dams that were not food restricted. In addition, male offspring of food restricted dams were less attractive and had fewer mating opportunities compared to that of male offspring of control male voles.

It is not known if the female offspring of food-restricted meadow vole dams also display similar persistent deficits in growth and behavior and if these effects are manifested only if their mothers were food restricted earlier rather than later during lactation. Thus, we tested the hypothesis that female offspring of meadow vole dams that were food restricted during early lactation (days 1-7), middle lactation (days 8-14), and late lactation (days 15-21) show persistent, negative effects on their body mass at weaning and display deficits in the components of sexual and copulatory behavior as adults. To do so, we measured the body mass and the three components of sexual behavior and copulatory behavior of female offspring: attractivity, proceptivity, and

receptivity. Attractivity, for meadow voles, like many other terrestrial mammals, refers to the appeal of a female's odors and scent marks to male conspecifics (Pierce et al., 2005). Proceptivity includes the behaviors displayed by females to show interest in and to facilitate interactions with males, such as investigating the scent marks of potential mates (Johnston, 1979; Pierce et al., 2005; Hobbs & Ferkin, 2012). Attractivity and proceptivity establish communication and allow females to coordinate behaviors that facilitate or inhibit direct interactions with potential mates (Beach, 1976; Stopka & Macdonald, 1998; Ferkin, 2011). Receptivity is characterized by a female's willingness to mate (Beach, 1976; Pierce et al., 2005; Sabau & Ferkin, 2013a). In females, lordosis is an indicator of a female's willingness to mate (Gray & Dewsbury, 1975). We considered female voles to be receptive if they allowed a male to mount, intromit, and ejaculate (delBarco-Trillo & Ferkin, 2007; Sabau & Ferkin, 2013a). Because we found that the male offspring manifested persistent deficits in growth and sexual behavior if their mothers were food restricted in middle lactation (Sabau & Ferkin, unpublished data), we predicted that maternal food-restriction during middle lactation will induce similar adverse effects in growth and sexual behavior of their female offspring.

METHODS

Animals

We used meadow voles that were 3rd to 4th generation descendants of free-living voles captured in New York, USA. The voles were born and raised under a long photoperiod (14:10 h, L: D, lights on at 0700h CST). Voles used in this study had been housed singly in clear plastic cages (27 x 16.5 x 12.5 cm, l x w x h) for 4 weeks prior to

its start. These voles had continuous access to water, food (Harlan Teklad Rodent Diet, #8640, Madison, WI, USA); this diet contains 22% protein. We followed Animal Care Protocol 0647, which was approved by the IACUC at The University of Memphis. We adhered to the 'Guidelines for the use of animals in research' as published in *Animal Behaviour* (1991, 41:183–186) and the laws of the country where the research was conducted.

We used female offspring of dams from the previous study by Sabau and Ferkin (2013b). In that study, Sabau and Ferkin (2013b) randomly assigned day-1 lactating female meadow voles to one of the four groups of 11 dams each. These four groups were comprised of dams that had continuous access to food throughout lactation (control), and dams that were provided with 70% of the daily intake of the control dams between day 1 and 7 (food restricted, FR 1-7), between day 8 and14 (FR 8-14), and between day 15 and 21 of lactation (FR 15-21) (Sabau & Ferkin 2013b). Dams in the FR groups had continuous access to food on days when they were not food restricted. For example, dams in treatment group FR 1-7 were provided with 70% of the daily intake of control dams between days 1-7 of lactation but had continuous access to food between days 8-21 of lactation. On day 22 of lactation, the pups from all four groups were weaned, housed with littermates in separate cages. No statistical differences existed in the number of male and female pups that were weaned per litter per treatment (4.2 ± 0.5 pups per litter; Sabau & Ferkin, 2013b). When the pups were 34 days-old, they were separated from littermates, and housed individually in clear polycarbonate cages (27 × 16.5 × 12.5 cm, l x w x h).

Body Mass of Female Offspring

Female offspring of dams from the FR 1-7, FR 8-14, FR 15-21 and female offspring of dams from the control group were weighed to the nearest of 0.1 gram (Ohaus GT4000 Automatic Balance, Florham Park, NJ) every 3-5 days when they were between 22 and 43 days old, and every 10 days thereafter until they were 98 days old. In this statistical analysis, litter identity was the random factor because the unit of replication was the treated mother not the individual offspring. We used the average body mass of females in each litter to determine if the mean body mass of female pups differed between treatment groups. We used separate 2-way repeated measures ANOVA to determine whether significant differences existed in the body mass of the female offspring in the three different FR groups and in the control group. If statistically significant differences were revealed, we conducted 1-way ANOVA's followed by Holm-Sidák post hoc pairwise comparisons to determine the significant treatment effects.

Food Intake of Female Offspring

The food intake of females from the FR 1-7, FR 8-14, FR 15-21 groups and the control group was monitored until they were 98 days old. Briefly, 30 grams of food was placed into the cage-lid hopper of each female. Twenty-four hours later, we removed the female from its cage and weighed any food that remained in the cage-lid hoppers and on the floor of the cage to determine the daily food intake. We used separate 2-way repeated measures ANOVA, followed by separate 1-way ANOVA's, and Holm-Sidák post hoc pairwise comparisons to determine the significant treatment effects.

Sexual Behaviors

We began testing the female offspring of the dams in the FR 1-7, FR 8-14, FR 15-22, and female offspring of the control dams for sexual behavior (attractivity, proceptivity, and receptivity) when they were 60-66 days old. The female voles underwent a single attractivity, proceptivity, and receptivity test. We used males and females that were unfamiliar and unrelated to the voles with which they were tested; these males were not reared by mothers that had been food restricted. We did not use more than two individuals from the same litter in any test to eliminate the potential for litter effects. We used a Latin Squares design to allow female voles to serve as scent donors in the attractivity tests and then as subjects in the proceptivity tests (Pierce et al., 2005). That is, some females were subjects in the attractivity tests first, some were subjects in proceptivity tests first, and others were first used as donors in attractivity tests. All female subjects and scent donors were tested for receptivity last. A minimum of 3 days separated successive tests with the same vole.

Attractivity Component

Scent donors were 18 female offspring of dams from the control group and 12 female offspring from each treatment of the dams from the FR 1-7, FR 8-14, and FR 15-21 groups. The FR female offspring and female offspring in the control groups were used as scent donors once. Subjects were 36 male voles that had continuous access to food and were 120-150 days of age, born and raised in long photoperiod, and housed singly for 30 days prior to testing. Males were randomly chosen from a pool of 68 sexually experienced voles that were unrelated to and unfamiliar with the females used in

the attractivity tests; these males had sired a litter 30-50 days before being used as subjects.

We used male subjects to compare the attractivity of scent marks of daughters of food restricted dams and that of daughters of control dams. Each male subject underwent a single 10-minute attractivity test that followed the procedures detailed elsewhere (Pierce et al., 2005; Sabau & Ferkin, 2013a). Briefly, we recorded the amount of time in seconds that males spent licking or sniffing (the subject's nose comes within approximately 1-2 cm) the anogenital area scent mark of the each female donor in the following pairings: 1) a female offspring of a dam from the FR 1-7 group versus a control female, 2) a female offspring of a dam from the FR 8-14group versus a control female, and 3) a female offspring of a dam from the FR 15-21 group versus a control female. The test began when the slide was placed into the cage of the male subject.

We used a clean, glass microscope slide (2.5 x 7.6 cm) that contained the scent marks of two female donors to be presented to the subjects (Pierce et al., 2005; Sabau & Ferkin, 2013a). The test slide was a clean, glass microscope slide (2.5 x 7.6 cm) that contained the scent marks of two female donors. Each slide was divided in three equal sections. Each section was 2.5 cm long. One end section of the slide contained a scent mark of a female donor that was reared by a food-restricted dam, while the other end section of the slide contained a scent mark of a female that was reared by a control dam. The middle section contained no scent marks. We used anogenital area scent marks because they are sexually discriminable and are deposited by voles in the runways and near their nests (Ferkin et al., 2004). Briefly, the anogenital area of a female donor was rubbed for approximately 5 seconds against the left- or right side of a clean slide. The

position of the two scent marks was alternated on the left- or right-side of the slide for each test. After both scent marks had been placed on the slide, we suspended the slide on a clip and hook apparatus 1 cm above the substrate in the home cage of the male subject (Pierce et al., 2005). The experimenter wore disposable latex gloves to minimize human scent transfer while handling all slides. The investigator recording the behaviors was blind to the treatment conditions of the female voles in the attractivity tests, as well as the proceptivity and receptivity described below.

We used matched-paired t-tests to determine whether significant differences existed in the amount of time each subject spent investigating the scent marks of the two donors in the attractivity test (Pierce et al., 2005). Female voles were considered to produce more attractive scent marks if males spent significantly more time investigating their mark relative to that of another female (Pierce et al., 2005; Sabau & Ferkin, 2013a). Significant differences were accepted at $\alpha < 0.05$ for all statistical tests. We used SPSS 13.0 to analyze the data.

Proceptivity Component

The proceptivity test followed the details used by Pierce et al. (2005) and Sabau and Ferkin (2013a). The procedure for this test is similar to that of the attractivity test with these notable exceptions. First, scent donors were 48 female offspring and 48 male offspring that had been reared by dams that were not food restricted; the male and female scent donors were between 120-150 days of age. The subjects were female offspring of dams from the FR 1-7, FR 8-14, FR 15-21 and those of dams from the control group; there were 12 different females per group). Second, during the 10-minute proceptivity

tests, we recorded the amount of time female subjects investigated a slide containing the scent mark of the male scent donor and the other end of the slide containing the scent mark of the female scent donor. Each female subject was tested once during the proceptivity test. We used matched-paired t-tests to determine whether significant differences ($p < 0.05$) existed in the amount of time each subject spent investigating the scent marks of the male and female scent donors. Female subjects were considered to display proceptive behavior by spending significantly more time investigating the scent mark of the male scent donor than that of the female scent donor (Pierce et al., 2005).

Receptivity Component

We used the same methods for testing receptivity described by Pierce et al. (2005) and Sabau and Ferkin (2013a). Briefly, a sexually naïve female vole offspring of dams from either the control group or those of dams from one of the three FR groups was placed in a clear, plastic cage (37 × 21 × 15 cm; l, w, h), containing a sexually experienced, 100-150 day-old, unfamiliar male vole, hardwood shavings, nesting material and water. We paired 48 males and 48 female voles (n = 12 females from each of the three FR groups and 12 females from the control group). We allowed each pair to interact for 4 hours; meadow voles typically mate within this 4-h period (delBarco-Trillo & Ferkin, 2004; Vaughn et al., 2008, 2011). We recorded each 4-hour pairing with a Sony Handycam DCR-SR68. During playback, we scored whether or not the females mated. We considered the female to be sexually receptive if she allowed the male to ejaculate at least once (delBarco-Trillo & Ferkin, 2007). We used binomial tests to compare the

number of female offspring from each of the three FR groups that mated against the number of female offspring from the control group that mated.

Copulatory Behavior

We did not measure the amount of time that female meadow voles were in lordosis. For meadow voles, lordosis by females and mounting by males and are highly correlated (delBarco-Trillo & Ferkin, 2007). We recorded the following variables: the latency to first mounting by the male, the latency to first ejaculation, the total number of ejaculations by each male, and the amount of time from the beginning of the first copulation and ejaculation to the end of the last copulation and ejaculation. We used separate one-way ANOVAs to determine if differences among female offspring existed in these variables. Group differences were identified using Tukey's multiple paired comparisons.

RESULTS

Body Mass of Female Offspring

The body mass of female offspring was affected by food restriction during lactation ($F_{3, 34}$= 6.0.7, $p < 0.005$) and whether food restriction occurred during early, middle or late lactation ($F_{3, 103.7}$= 269.7, p =0.0001). A significant interaction existed between these variables ($F_{9.1, 103.7}$= 2.98, p = 0.003). With the exception of when the females were 26 days of age or 34 days of age, the body weights of female offspring of FR 1-7 dams and FR 8-14 dams between days 21 and 48 were lower than the body weights of female offspring of FR 15-21 dams and those of control dams ($p < 0.05$; Fig. 1). During this time, there was no difference in the body weights of female offspring of

FR 15-21 dams and the female offspring of control dams. From day 49 to 98, however, the female offspring of the FR 1-7 dams weighed less than the female offspring of the FR 15-21 and control dams ($p < 0.05$; Fig. 1). During the is time period, no difference existed in the body weights of the female offspring of FR 8-14, FR 15-21, and control dams ($p < 0.05$; Fig. 1).

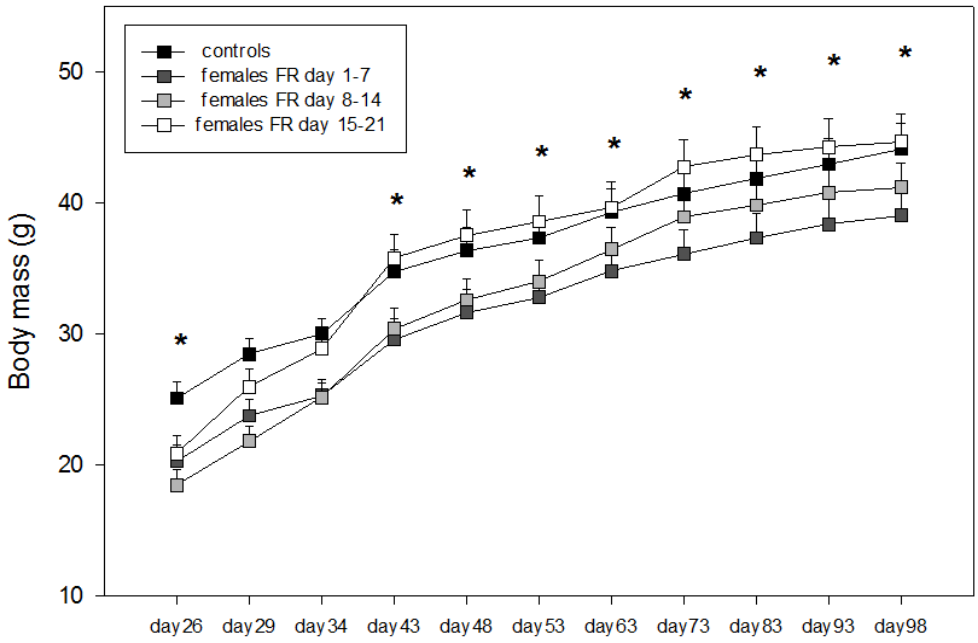


Figure 1. Mean ± SEM body mass (grams), of female offspring of FR 1-7 dams, FR 8-14 dams, FR 15-21 dams, and control dams. An asterisk (*) denotes significant differences between groups of female offspring ($p < 0.05$).

Food Intake of Female Offspring

The daily food intake between days 34 and 98 was similar for female offspring reared by control dams and for female offspring of FR 1-7, FR 8-14, and FR 15-21 dams ($F_{3, 34} = 1.57$, p =0.21). Daily food intake was also not affected by whether food restricted occurred during early, middle, or late lactation ($F_{8.9, 101.7} = 1.77$, p = 0.08). On day 34 the daily food intake was 4.5(± 0.3) grams for controls, 4.5(±0.5) for offspring of FR 1-7 dams, 4.9(±0.3) grams for offspring of FR 8-14 dams and 4.7(± 0.5) grams for offspring of FR 15-21 dams. The daily food intake increased slowly, such as at day 98 the daily intake for controls was 4.8(±0.6) grams, 5.2 (±0.5) grams for offspring of FR 1-7 dams, 5.7 (±0.6) grams for offspring of FR 8-14 dams and 5.3 (± 0.3)grams for offspring of FR 15-21 dams.

Attractivity

Maternal food-restriction during lactation affected the attractivity of scent marks of female offspring to opposite sex conspecifics (Fig. 2). Male voles spent significantly more time investigating the scent mark of female offspring of control dams than that of female offspring of FR 8-14 dams ($t_{11} = - 2.45$, p = 0.03; Fig. 2b). Also, males spent more time investigating the scent marks of control females over the scent of female offspring of FR 15-21 dams ($t_{11} = - 2.93$, $p = 0.01$; Fig. 2c). However, male voles spent similar amounts of time investigating the scent marks of female offspring of FR 1-7dams and that of control females ($t_{11} = - 0.59$, p = 0.56; Fig. 2a).

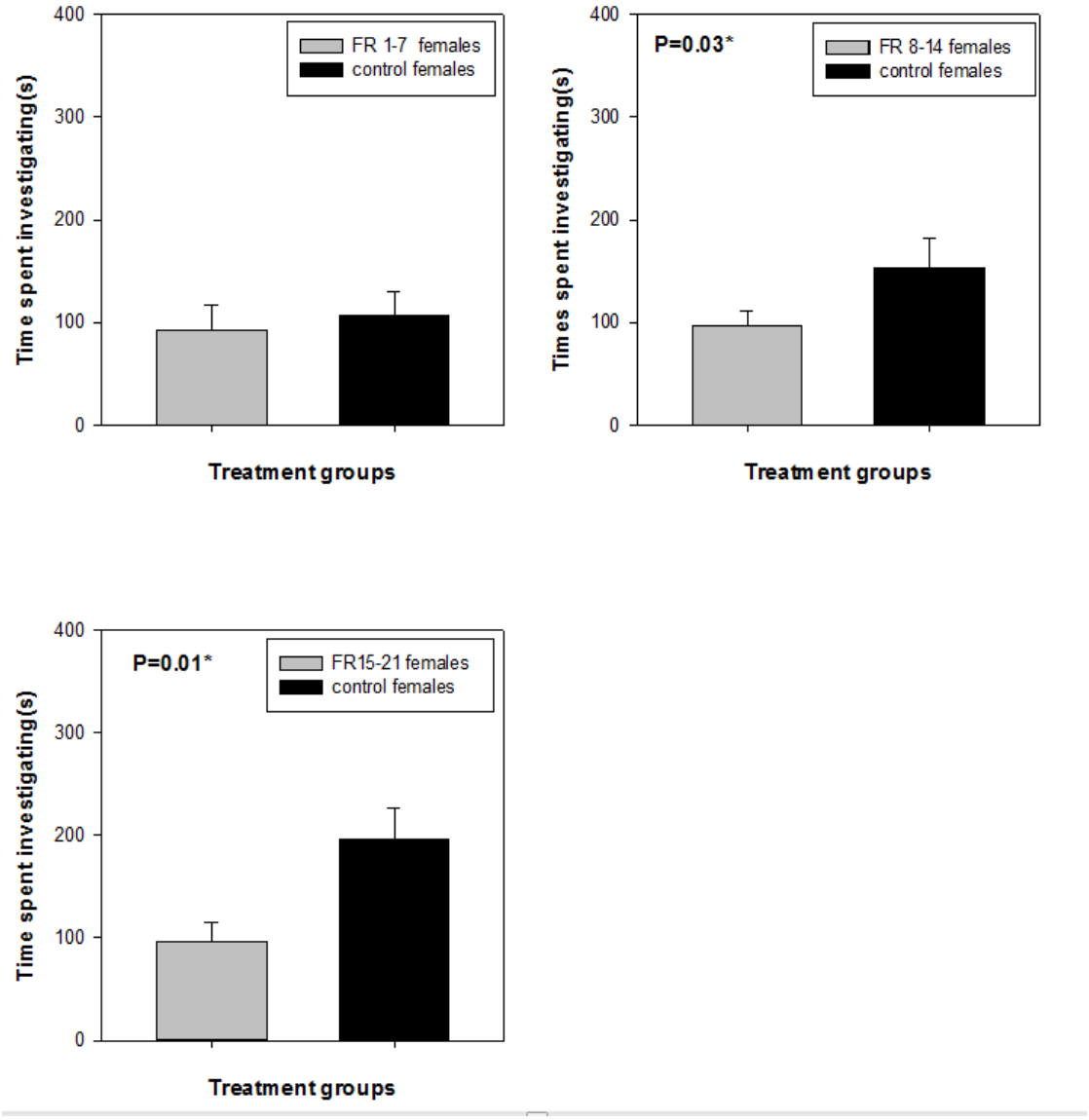


Figure 2. Mean ± SEM amount of time (seconds) spent by male voles investigating the anogenital scent marks during a 10-minute test in the following pairings of the female offspring of control and food restricted (FR) dams: control females versus FR 1-7 females; control females versus FR 9-14 females; control females versus FR 15-21 females. An asterisk (*) indicates significant differences between each pair ($p < 0.05$).

Proceptivity

Maternal food-restriction did not affect the proceptivity of their daughters. Female offspring of FR 1-7 (t_{11} = - 3.26, p = 0.00), FR 8-14 dams (t_{11} = -6.83, p = 0.00), FR 15-21 dams (t_{11} = - 4.77, p = 0.00) and control females (t_{11} = - 5.69, p = 0.00) spent more time investigating the scent mark of male conspecifics compared to that of female conspecifics (Fig. 3).

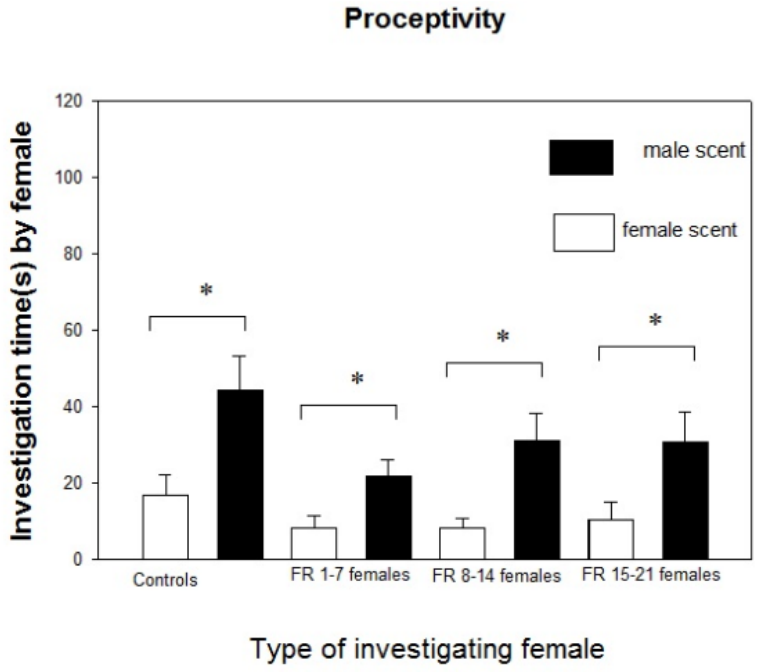


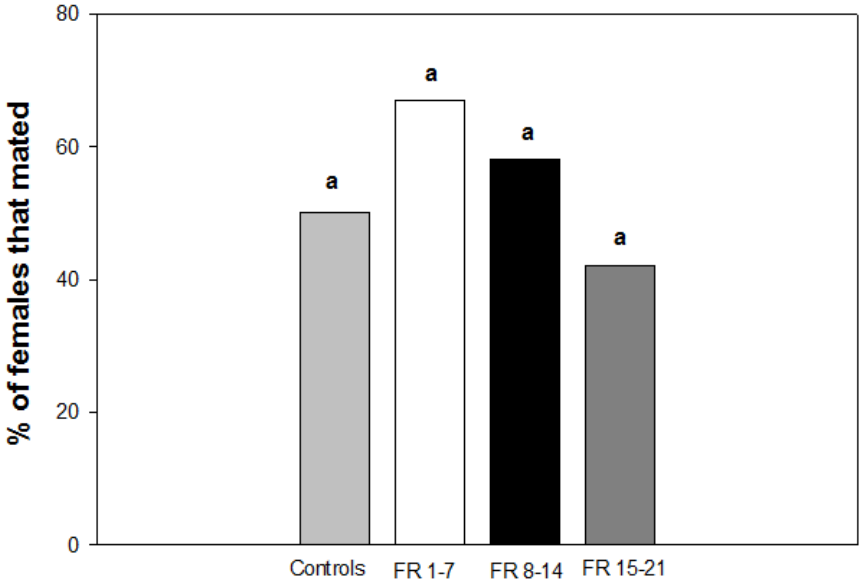
Figure 3. Mean ± SEM amount of time (seconds) spent by female offspring of control dams, FR 1-7 dams, FR 8-14 dams, and FR 15-21 dams investigating the anogenital scent marks of a male conspecific and a female conspecific during a 10-minute test. An asterisk (*) indicates significant differences between groups ($p < 0.05$).

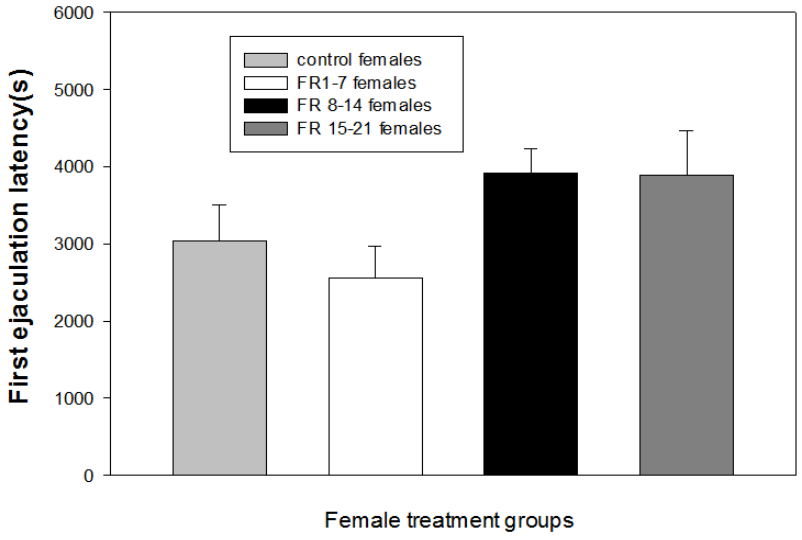
Receptivity

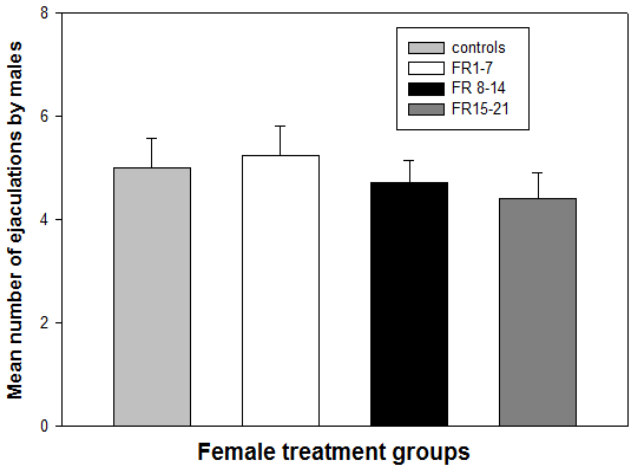
The proportion of females that mated with male voles during the 4 hour test was not affected by whether their mothers were food restricted during early, middle, or late lactation (Binomial Critical Value test, $p > 0.05$; Fig. 4a). Specifically, 8 of 12 female offspring of FR 1-7 dams, 7 of 12 female offspring of FR 8-14 dams, 5 of 12 female offspring of FR 15-21dams, and 6 of 12 control females copulated with the males.

Copulatory Behavior

The average time required to complete an ejaculatory series was shorter for daughters of FR 1-7 females than it was for daughters of FR 8-14, FR 15-22, and control dams ($F_{3, 25}= 7.27$; $p = 0.001$). Males that copulated with females from group FR 1-7 took in average 190.5 ±27.4(s) to complete an ejaculatory series, while for female offspring from group FR 1-7 took 100.1±79(s), for female offspring from group FR 8-14 took 236.4 ± 60.77 (s) and for female offspring from group FR 15-21 took 209.3 ±41.6 (s).There was also a significant difference in for total copulation interval ($F_{3, 25}= 4.26$; $p = 0.016$). The total time spent for copulation was shorter for female offspring of FR 1-7 dams than it was for those of female offspring of FR 8-14, FR 15-21 dams and control dams (Tukey's post hoc $p < 0.05$; Fig. 4). The males that mated with the females in the control group or in the FR groups had a similar number of ejaculations ($n = 5.3 \pm 0.6$ ejaculations; ($F_{3, 25} = 0.61$; $p = 0.61$) and did not differ in their latency to first mount (2520 ± 513.6) seconds; $F_{3, 25} = 1.89$, $p = 0.16$) or their latency to first ejaculation (3280.7 + 782) seconds ($F_{3, 25}= 2.51$, $p = 0.08$)


% of females that mated
80
60
40
20
0
a
a
a
a
Controls
FR 1-7
FR 8-14
FR 15-21


First ejaculation latency(s)
6000
5000
4000
3000
2000
1000
0
control females
FR1-7 females
FR 8-14 females
FR 15-21 females
Female treatment groups



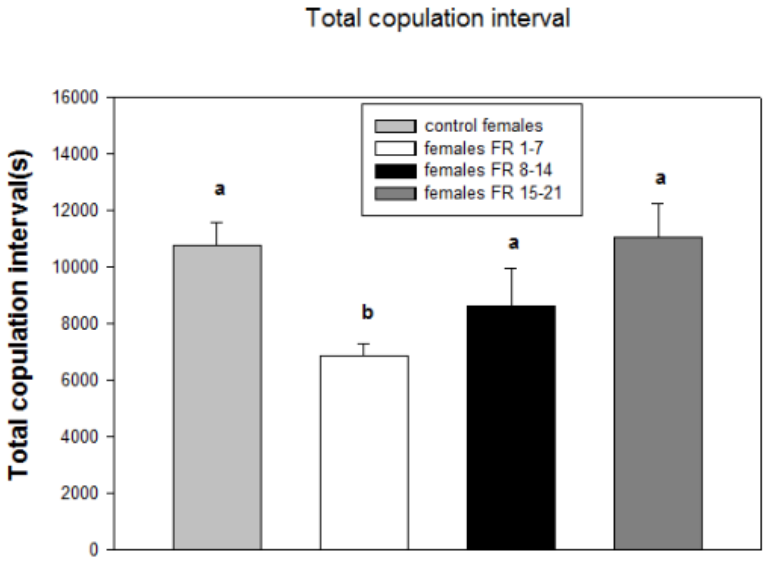


Figure 4. 4a) Percentage of female offspring of control dams, FR 1-7 dams, FR 8-14 dams, and FR 15-21 dams that mated when paired with a male vole for 4 hours. 4b) Mean ± SEM amount of time (seconds) to first ejaculation. 4c) Mean ± SEM number of ejaculations by the males when paired with offspring of control dams and the FR 1-7, FR 8-14, and FR 15-21 dams. 4d) Mean ± SEM amount of time (seconds) of the total copulation interval. Histograms capped with different letters indicate significant differences between groups ($p < 0.05$).

DISCUSSION

We found that food-restricted, lactating meadow voles weaned female offspring that displayed persistent and adverse effects on their attractivity relative to that of female offspring of lactating meadow voles that were not food restricted. The manifestation of these negative effects was dependent on the timing of food deprivation during lactation. Female offspring of FR 8-14 and FR 15-21 dams produced anogenital marks that were not as attractive as those produced by female offspring of control dams. In contrast, the scent marks of female offspring of FR 1-7 dams were as attractive as to males as were those of female offspring of control dams. A similar pattern emerges when we compare the results of this study to those obtained for the attractiveness of male offspring of FR dams (Sabau and Ferkin, unpublished data). In that study, sons of FR 8-14 dams produced scent marks that were not as attractive to females as those produced by sons of control dams. In contrast, the scent marks of male offspring reared by FR 1-7, FR 15-21, and control dams were similar in their attractiveness to female voles. This reduction in the attractiveness to the opposite sex may be reflect the effects of food restriction during middle lactation has on the development or secretions of glands and tissues the voles will use to create or deposit scent marks (Ferkin et al., 1991; Ferkin & Johnston, 1993).

Food restriction during lactation did not affect female's interest in opposite-sex conspecifics, as measured by our proceptivity test. Female offspring from all treatment groups spent more time investigating the scent marks of an adult male than those of an adult female. Similar results were shown for female rats that were food restricted from birth; these females showed no change in pre-copulatory proceptive behavior (hopping, darting, and presenting posture) at 80 days of age (Hlinák & Franková, 1983). Food

deprived and control female meadow voles over-marked and self-groomed at high rates when they were exposed to the scent marks of male voles (Hobbs et al., 2008, 2012; Hobbs & Ferkin, 2011). Our results also matched those from a study on the behavior of male meadow voles that were reared by food restricted dams (Sabau & Ferkin, unpublished data). In that study, male offspring of food-restricted dams and control dams spent similar amounts of time investigating the scent marks of an opposite sex conspecific. Given that proceptive behavior initiates or facilitates further sexual interactions with potential mates (Beach, 1976; Stopka & Mcdonald, 1998), any reduction in proceptive behaviors may reduce the likelihood of a female attracting mates or signaling her presence in an area (Hobbs & Ferkin, 2012). A similar argument can be made for the fact that male offspring of food-restricted, lactating dams did not alter their interest in the scent marks of female conspecifics (Sabau & Ferkin, unpublished data). These speculations may explain why a period of reduced food availability during development may not cause a reduction in the proceptive behaviors of meadow voles.

The receptivity of female voles was not affected by whether or not they were reared by food-restricted dams. Female voles in all our groups allowed a male to mount, intromit, and ejaculate. This result is interesting in that food restriction during lactation had an inhibitory effect on the receptivity of male offspring in meadow voles. Sabau and Ferkin (unpublished data) found that only 33% of the male offspring of FR 8-14 meadow voles dams mated compared to the 75% of the male offspring of FR 1-7 dams, 67% of the male offspring of FR 15-21 dams, and 73% of the male offspring of the control dams. However, no differences existed in copulatory behavior among the male meadow voles that did mate (Sabau & Ferkin, unpublished data). In rats, male offspring raised by food

restricted dams showed deficits in the mating success of male offspring (Larsson et al., 1974; Rhees & Fleming, 1981). The sex differences in mating success of offspring reared by food-restricted dams may have consequences for free-living voles and may represent a tradeoff between the survival of the pup and its future sexual behavior. Only a small proportion of male meadow voles mate and sire offspring (Boonstra et al., 1993; Berteaux et al., 1999). Our results suggest that male offspring of dams that were food restricted during middle lactation may be less likely to compete with male conspecifics for mates and have lower fitness. In contrast, most adult female meadow voles will mate (Boonstra et al., 1993; Berteaux et al., 1999). However, our findings suggest female offspring of dams that were food restricted during early, middle, or late lactation may mate but they may be a last option for males or may mate with less desirable male conspecifics (Pierce et al., 1990; Boonstra et al., 1993).

Maternal-food restriction during lactation affected the body weight of the female offspring of meadow vole dams. With the exception of when they were 26 and 34 days of age, the body weights of female offspring of FR 1-7 dams and FR 8-14 dams were lower than the body weights of female offspring of FR 15-21 dams and control dams up until 48 days of age. After 48 days of age, the body weight of female offspring of FR 1-7 dams was lower than those of the female offspring of FR 8-14, FR 15-21, and control dams. After 48 days of age, the body weight of female offspring of FR 8-14 dams were similar to the body weights the female offspring of the FR 15-21 and control dams. Similarly, Sabau and Ferkin (unpublished data) found that 21-48 day old male offspring of FR 1-7 dams weighed less than did those of FR 8-14, FR 15-21, and control dams. However, unlike their sisters, the male offspring of FR 1-7 dams caught up; by day 98 they weighed

the same as male offspring raised by FR 8-14, FR 15-21 and control dams (Sabau & Ferkin, unpublished data). Taken together, our findings for the offspring of food-restricted dams are similar to those studies showing that nutritional stress during gestation and lactation caused female rats and mice to wean offspring with lower body weight compared with the body weight of offspring reared by dams that were not under nutritional stress (Rossiter, 1996; Teixeira et al., 2002). Our results, however, differ from those of Meikle and colleagues, who found that maternal food deprivation during pregnancy was not sufficient to affect the body mass at weaning of female offspring (Drickamer & Meikle, 1988; Meikle & Westberg, 2001). In contrast, our results show that for male and female offspring of FR 1-7 meadow vole dams have lower body weights than do their counterparts, but the relatively lower body weight persists into adulthood for female voles. This long-term effect on females could affect their lifetime reproductive success and survival. It has been shown repeatedly for mammals that lower body mass is positively associated with decreased survival, smaller litters, and lower reproductive success (Sauer & Slade, 1986, 1987; Guzman, 2006). For example, rhesus monkeys that were born at a lower body mass gave birth to daughters that had low body mass (Price et al., 1999).

In meadow voles, food restriction during lactation was sufficient to induce persistent deficits in the growth and sexual behavior of male and female meadow voles. Although there is no clear functional pattern to explain the sex differences in the phenotypes of the offspring that were reared by dams that were food restricted during lactation, most adverse effects in meadow voles that we have observed are triggered by events associated with being reared by a FR 8-14 dam. We offer the speculation that

female and male voles have different windows of vulnerability to maternal food-restriction during lactation and that middle lactation is a period of time when offspring are sensitive to food restriction. During middle lactation, the offspring open their eyes, become more mobile, readily leaving and returning to the nest, and begin to eat solid food (Nadeau, 1985). Coincident with these developmental events in the pups is a gradual and steady decline in the amount of time dams dedicate towards maternal care (Libhaber & Eilam, 2004; McGuire & Novak, 1984; Hayes & Solomon, 2006, 2007). The deficits seen in FR 8-14 offspring may be associated with a more pronounced reduction in nursing, mother-pup interaction, and licking experienced by pups that were reared by FR 8-14 dams than by pups reared by FR 1-7, FR 15-21, and control dams (Sabau & Ferkin, 2013b). Studies have suggested that female Mongolian gerbils and prairie voles (*M. ochrogaster*) may respond differently to the male and female pups (Clark et al., 1990; Hayes & Solomon, 2006, 2007). However, we do not know if food-restricted meadow vole dams differ in the amounts of maternal care they direct towards their sons or daughters and whether it varies across lactation. Alternatively, the deficit in the attractivity of the male and female offspring of FR 8-14 dams may be due to the fact that these offspring were also experiencing food restriction at a time when they were beginning to eat solid food. In any case, it appears that a reduction in maternal care or the food restriction experienced by the pups could singly or together be sufficient to trigger persistent, sex-specific effects on the body mass and sexual behavior of male and female meadow voles.

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CHAPTER 6

CONCLUSIONS

Meadow voles, *Microtus pennsylvanicus*, live in transitional grasslands where female voles may face limited food availability during pregnancy, postpartum estrus period (PPE) and lactation (Bergeron & Jodoin 1987, 1989; Madison 1980). In chapter 2, we tested the hypothesis that food deprivation (FD) and restriction (FR) during late gestation causes deficits in the attractivity, proceptivity, and receptivity of females when they enter PPE. On day 1 of lactation, females that were FD and FR were less receptive to males and produced scent marks that were no longer as attractive as those produced by control PPE females and PPE. Food deprivation but not FR caused females to no longer display preferences for the scent marks of males (proceptivity). Dams facing food deprivation or restriction during late gestation may have to balance the benefits of mating during PPE with the increased costs associated with raising multiple litters under nutritional stress.

When females face food restriction during lactation, they may have to choose between altering the amount of maternal behavior they provide to their young, reducing the size of their litter, or not adjusting their behavior or litter size (Smart & Preece 1973; Smart 1976; Marsteller and Lynch 1987; Perrigo 1987, 1990). How females allocate energy to maternal investment may depend on the energy costs of different lactation stages. In chapter 3, we hypothesized that the amount of time female voles provided maternal behavior would differ if they were deprived of food during early, middle, or late in lactation. We tested this hypothesis by placing lactating female meadow voles (dams) into one of four groups: dams that underwent a 30% caloric restriction during days 1-7 of

lactation, those that were food restricted on days 8-14, those that were food restricted on days 15-21, and dams that did not undergo food restriction during lactation. Dams that were not food restricted spent more time engaged in maternal behavior than dams that were food restricted during lactation. Dams that were food restricted during days 8-14 of lactation displayed the most pronounced decline in maternal behavior relative to dams that were restricted during days 1-7 or days 15-21 of lactation. This effect was most dramatic in the amount of time that dams spent licking their pups. Reduced licking of pups may affect the mother-pup bond, inducing pups to possibly develop deficits in their social and sexual behavior as adults. The results also suggest that when they are faced with a food shortage, particularly during the first week of lactation, lactating female meadow voles do not reduce the size of their litter but do show a decrease in maternal behavior toward pups. The results from chapter 3 showed that the effects of maternal FR were more striking in the amount of time that dams spent licking their pups. Reduced licking of pups may affect the mother-pup bond, inducing pups to possibly develop deficits in their social and sexual behavior as adults. Thus, in chapter 4, we tested the hypothesis that male offspring of female meadow voles that were 30% food restricted (FR) during days 1-7 of lactation (FR 1-7), days 8-14 of lactation (FR 8-14), or late days 15-21 of lactation (FR 15-21) lactation show persistent, negative effects on their sexual behavior as adults relative to male offspring of females that were not food restricted. We measured three components of sexual behavior, attractivity, proceptivity and receptivity, beginning when the males were 98 days of age. Food restriction during middle lactation (FR 8-14) but not during early (FR 1-7) and late lactation (FR 15-21) was sufficient to induce adult male voles to produce anogenital marks that were not as attractive as those

produced by control males. Food restriction during lactation did not affect the proceptive behavior of male voles but did affect their receptivity. Only 4 of 12 FR 8-14 male voles mated compared to 9 of 12 FR 1-7 males, 8 of 12 FR 15-21 males, and 8 of 11 control males. However, no differences existed in their copulatory behavior among the males that did mate. The body weight of FR 1-7 and FR 8-14 males was lower than that of FR 15-21 and control males when they were between 22 days of age (weaning) and 48 days of age (puberty) but was similar when the males were 98 days of age. Food intake was similar for the FR and control males between day 22 and day 98. It remains unclear, however, if this type of maternal effect on male offspring represents strategic programming of offspring behavior in response to the environment experienced by mothers or is a product of developmental processes of food restriction prior to weaning (Forstmeier et al. 2004).

In chapter 5, We tested the hypothesis that female offspring of meadow vole dams that were 30% food restricted (FR) during days 1-7 of lactation (FR 1-7), days 8-14 of lactation (FR 8-14), or days 15-21 of lactation (FR 15-21) show negative effects on their food intake, growth, and the three components of sexual behavior (attractivity, proceptivity, and receptivity) as compared with female offspring of control dams. Female offspring of FR 8-14 and FR 15-21 dams produced odors that were less attractive to males than odors produced by those of FR 1-7 and control dams. Female offspring of FR dams and control dams did not differ in their measures of proceptivity and receptivity. However, the total amount of time allocated for copulation was shorter for female offspring of FR 1-7 dams than the other female offspring. The results of this study coupled with those found in a study conducted on the male offspring of FR dams indicate

that food restriction during days 8-14 of lactation induces deficits in sexual behavior and body mass.

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APPENDIX

THE UNIVERSITY OF MEMPHIS

IACUC PROTOCOL ACTION FORM

To:	Michael Ferkin
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	7-22-2011

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 0700(Sperm Competition and Sex)

☒ Your proposal is approved for the following period:

From: July 22, 2011 To: July 21, 2014

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Revision Memorandum dated _____ for the following period:

From: _____ To: _____

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Revision Memorandum dated _____